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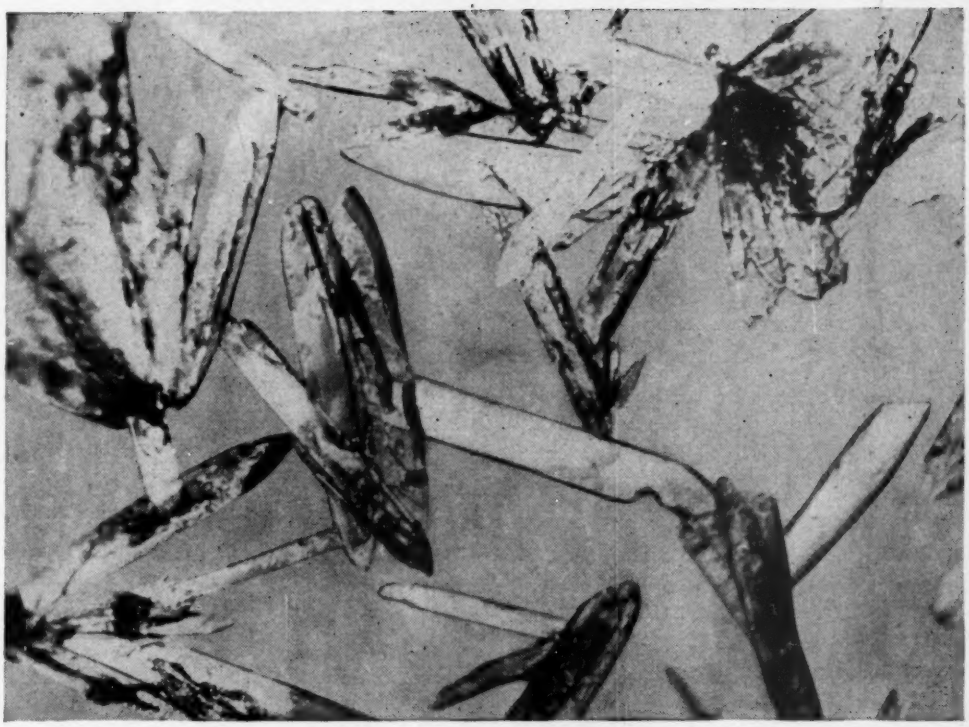

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The Recovery of Streptococci Associated with Bovine Mastitis from the External Surface of the Teat

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Blacksburg, Virginia

SINCE the spread of streptococcic mastitis still presents several unknown factors and since other workers, namely, Harrison[1] and Spencer,[2] have shown that the organisms concerned can establish themselves on the hands of milkers, it was thought desirable to determine if *Streptococcus agalactiae* could be recovered from the external surface of the teat prior to milking. Earlier work by Little[3] and Minett[4] indicated *Str. agalactiae* was an obligate organism, proliferating only in the udder and its secretions. Should it be found that *Str. agalactiae* can establish itself on the external surface of the teats, this would be one more factor to consider in controlling the spread of the infection in a herd.

In the experiments herein reported, no cow had been milked for at least eight hours prior to the time of collecting the specimen and no animal was used that showed evidence of a weak sphincter muscle. No collections were made if it was felt that there would be any other unusual likelihood of secretions from the gland contaminating the external surface of the teat.

MATERIALS AND METHODS

It was necessary to determine a suitable bacteriologic medium for this work since all specimens would be collected in barns where contamination would occur even under the most favorable conditions. Several different mediums containing various inhibiting agents were tried with varying results. The medium finally decided on was one very similar to that developed by Spencer[2] and will be referred to in this paper as inhibitor agar. The base medium had the following composition:

Veal infusion broth	400	cc.
Bacto-Peptone	5	Gm.
Sodium chloride	2.5	Gm.
Mannitol	5	Gm.
Agar	7.5	Gm.

The medium was prepared in the usual manner, the pH adjusted to 7.2 to 7.4, filtered through cotton gauze, and autoclaved at 15-lb. pressure for twenty minutes. After cooling to between 45 and 50 C., the following were added:

Sterile defibrinated bovine blood	5 per cent
Sterile 0.02 per cent aqueous solution of crystal violet	1 per cent
Sterile 0.5 per cent aqueous solution of bromcresol purple.	1 per cent
Sterile 0.5 per cent aqueous solution of sodium azide	1 per cent

The procedure for collecting the specimens consisted of first washing the udder thoroughly with cool water to avoid stimulating milk secretion more than necessary. The udder, teats, and adjacent areas were then washed with a 0.5 per cent solution of roccal, one of the commonly used dairy antiseptics, care being taken to avoid rough manipulation of teats. The end of the teat was then wiped with 70 per cent ethyl alcohol, a layer of sterile flexible collodion applied, and sufficient time allowed for drying. The teat was then washed thoroughly with sterile saline, using a sterile towel. Sterile gloves were used while the teat was scrubbed with a sterile brush and 60 to 175 cc. of sterile milk, the milk washings being collected in a sterile 400-cc. beaker and placed in two sterile bottles. Washing of the udder was then repeated using the antiseptic, the collodion removed, and quarter samples of milk collected in the usual way.

It was realized that in work of this nature

From the section on animal pathology, Virginia Agricultural Experiment Station.
Dr. Ingalls resigned Dec. 31, 1946.

precautions must be taken to avoid getting secretions from the udder into the milk washings. For this reason, the teat was examined carefully after the collodion had been applied and sufficient time had passed for it to dry to determine that a complete seal had resulted. When the scrubbing had been completed, the teat was wiped with an antiseptic solution and squeezed lightly to determine if the seal was intact. By the application of this technique, it was thought that the possibilities of getting udder secretions into the washings would be reduced to a minimum.

The specimens were taken to the laboratory and 9.5 cc. of the milk washings were removed from one bottle and added to 0.5 cc. of 0.5 per cent aqueous solution of bromcresol purple in a test tube. This tube and the unopened bottle were incubated at 37 C. The bottle from which the sample had been removed was held in a refrigerator at 10 C.

Sixteen to eighteen hours later stained films were made from each of the above and were examined microscopically for the presence of streptococci. Petri dishes containing 6 per cent ox blood nutrient agar and the inhibitor agar were then streaked. After twenty-four hours' incubation, colonies suspected of being streptococci were inoculated into veal infusion broth and further identification carried out on the basis of their biochemical reactions.

Serologic identifications by Lancefield's micromethod (group B) were also done on most cultures. The quarter milk samples collected were examined in the same way.

The various mediums used in identifying the organisms were made up as described by Plastridge.[5] The Streptococcus grouping serum used was obtained from a commercial laboratory. In the biochemical identification of the streptococci, the reactions considered indicative of *Str. agalactiae* were as shown in table 1.

The results obtained from 38 examinations by the method described above are summarized in table 2.

TABLE I—Reactions Considered Indicative of Streptococcus Agalactiae

Mannitol	Lactose	Arabinose	Raffinose	Salicin	Sorbitol	Inulin	Trehalose	Litmus milk	Aesculin	Sodium hippurate
No acid	Acid	No acid	No acid	Acid occasionally	No acid	No acid	Acid	Acid coagulation, partial reduction	Not fermented	Hydrolysis

DISCUSSION

A total of 38 complete examinations were conducted, and streptococci commonly recognized to be of a pathogenic nature in bovine mastitis were recovered ten times from the external surface of the teat.

Of the 38 examinations 10 were conducted on quarters not shedding streptococci in the milk at the time of collection. In no case were *Str. agalactiae* or *Streptococcus dysgalactiae* recovered from the external surface of the teat of a quarter not shedding streptococci.

Fourteen examinations were made of 1 cow in the station's experimental barns. This animal was shedding *Str. agalactiae* from the right rear quarter only. The cow was hand-milked regularly, and the organisms were recovered from the milk washings in eight of 14 trials. A study of the data on this animal reveals that *Str. agalactiae* was recovered from the external surface of the teat after an interval of two and a half and five days following the last milking before her dry period. However, the organisms were not recovered forty days after the last milking. In one trial, an attempt to recover the organisms from the external surface of a teat of a non-infected quarter of an infected udder was unsuccessful.

Thirteen examinations were carried out in a herd milked by machine. Seven of these were conducted on cows not shedding streptococci. In six attempts to recover the organisms from the milk washings of teats of infected quarters, *Str. agalactiae* was recovered once and *Str. dysgalactiae* once. *Str. dysgalactiae* was also recovered from the milk of this cow.

One attempt was made in another hand-milked herd where only 1 cow was affected. Significant streptococci were not recovered from the outside of the teat of the infected quarter.

In 28 of the 38 examinations, the teat was sealed with collodion. In 2 cases the collodion seal was unsatisfactory, as it had loosened before the washing was completed. Streptococci were recovered seven times

when the sealing of the teat was considered to be satisfactory.

SUMMARY

1) *Streptococcus agalactiae* was recovered nine times and *Streptococcus dysgalactiae* once in 28 attempts to recover the organisms from the external surface of the teats of infected quarters. In all cases, a minimum interval of eight hours had elapsed from the time of milking. The organisms were recovered as late as the fifth day following the last milking.

2) *Streptococcus agalactiae* was not recovered from the outer surface of teats of cows that were not shedding the organisms in their milk.

3) The procedure followed in cleaning the udder and teats, although more thorough than commonly recommended in the usual preventive measures for the control of mastitis, apparently did not in all instances destroy the organism on the external surface of the teat.

4) Evidence is presented that *Str. aga-*

TABLE 2—Data on Recovery of Streptococci from Teat Washings

Date of collection 1946	Ident. of animal	Quar. of udder scrubbed	Teat sealed with collodion	Quar. shedding Strepto-coccus	Gross changes in milk of quar. scrubbed	Species of Strepto-coccus re-covered in milk washings	Comments
5/16	#6	R.R.	No	Yes	None	None	
5/22	#6	R.R.	No	Yes	None	Agalactiae	
5/28	#6	R.R.	No	Yes	None	None	
6/4	#6	R.R.	No	Yes	None	None	
6/13	#6	R.R.	No	Yes	None	Agalactiae	
6/24	#6	R.R.	No	Yes	None	Agalactiae	
7/25	#52B6818 (normal)	R.F.	No	No	None	None	
7/29	#52B6818 (normal)	R.F. & R.R.	No	No	None	None	
8/13	#52B6818 (normal)	R.F.	No	No	None	None	
8/14	#6	R.R.	Yes	Yes	None	None	
8/16	#6	R.F.	Yes	No	None	None	
8/26	#6	R.R.	Yes	Yes	None	Agalactiae	
8/27	#6	R.R.	Yes	Yes	None	Agalactiae	
8/28	#6	R.R.	Yes	Yes	None	Agalactiae	
9/10	#6	R.R.	Yes	Yes	None	Agalactiae	Not milked for 2½ da. (dry).
9/17	#6	R.R.	Collodion loosened in scrubbing	Yes	None	Agalactiae	Not milked for 5 da. (dry).
9/11	Rex	L.R.	Collodion loosened in scrubbing	No	None	None	
9/11	Kate	L.F.	Yes	No	None	None	Previous history of mastitis.
9/18	Kate	L.F.	Yes	No	None	None	
9/18	Brilliant	R.F.	Yes	Yes	Slight	None	
9/18	Lass	R.F.	Yes	Yes	Slight	None	
9/25	Kate	L.F.	Yes	No	None	None	
9/25	Brilliant	R.F.	Yes	Yes	Slight	None	Gloves not used; but teat not touched by hands.
9/25	Lass	R.F.	Yes	No	None	None	
10/8	Roxie	R.F.	Yes	Yes	None	None	
10/8	Lass	R.F.	Yes	No	Abnormal milk; about dry	None	
10/8	Brilliant	R.F.	Yes	Yes	Slight	Agalactiae	
10/18	#6	R.R.	Yes	Cow dry	Dry	None	Dry 40 da.
10/29	#7 (Mennitt Herd)	L.F.	No	Yes	Yes	None	
11/12	Ruby	L.R.	Yes	Yes	None	None	
11/12	Spot	L.R.	Yes	No	None	None	
11/12	Fannie	R.R.	Yes	Yes	None	Dysgalactiae	
11/20	Ruby	L.R.	Yes	Yes	None	None	
11/20	Fannie	R.R.	Yes	(?)	None	None	Samples drawn from quar. lost due to mishap.
11/20	Kate	R.R.	Yes	(?)	Slight	None	
12/4	Ruby	L.R.	Yes	Yes	None	None	
12/4	Fannie	R.R.	Yes	Yes	None	None	
12/4	Kate	R.R.	Yes	Yes	None	None	

lactiae will remain on the external surface of the teat for at least eight hours even with the procedures used. This work also indicates that this organism may become established deep in the wrinkles of the teat skin and remain there for several days.

5) These findings suggest that further work to determine the importance of these streptococci on the surface of the teat in both hand-milked and in machine-milked herds is desirable.

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Correction

In the July, 1947, issue of the *American Journal of Veterinary Research* (p. 311-313), Dr. Hans Elias' paper entitled "Comparison of Duodenal Glands in Domestic Animals" was erroneously identified as "from the U. S. Public Health Service, Atlanta, Ga." Dr. Elias is a project supervisor in the U. S. Public Health Service, but his contributions to the JOURNALS of the AVMA are not official contributions of the Service. They are research work done on his own time and financed by himself. The laboratories in the Department of Zoölogy of the University System of Georgia in Atlanta are used in this work.

Efficacy of Intramammary Infusion with Penicillin and Diluents on Streptococcic Mastitis

G. R. SPENCER, D.V.M., M.S., MARY E. KRAFT, B.S., and G. K. L. UNDERBJERG, D.V.M, Ph.D.

Madison, Wisconsin

EXTENSIVE studies have shown that penicillin is a safe agent for intramammary infusions. It has also been found to be efficacious in the treatment of streptococcic mastitis, but the results of different workers have varied considerably. Porter *et al.*,[1] using a single infusion of 1,000 to 100,000 Oxford units in 500 cc. of distilled water, cured from 37 to 63 per cent of the treated quarters. Subsequently, Porter and Kelman[2] indicated that the volume of diluent had little effect on treatment. A solution of penicillin in 10, 100, or 500 cc. of water gave approximately the same results with each volume.

Schofield[3] employed oils as carriers for penicillin but with unsatisfactory results due to difficulty in making a suitable suspension. Later Schofield and Barnum[4] freed two out of three quarters from streptococcic infection with an infusion of 100,000 units of penicillin in oil. Walker[5] used a beeswax-peanut oil suspension of penicillin for staphylococcal mastitis and reported the majority of quarters were freed of infection. Bryan[6] reported the effective use of solid mastics as carriers of penicillin for intramammary treatment of mastitis. Johnson and Fincher[7] prepared an emulsion of an aqueous solution of penicillin in mineral oil and treated successfully six quarters of 3 cows. Schalm and Casselberry[8] found mineral oil had little advantage over distilled water as a carrier for penicillin. No method of preparation of the mixture of mineral oil and penicillin was indicated.

The reports on the oils as carriers suggested additional trials to determine the comparative efficacy of peanut oil, mineral oil, and distilled water as carriers for penicillin in the therapy of bovine mastitis.

MATERIALS AND METHODS

The penicillin was obtained from two lots. One lot was a bulk calcium salt with a potency of 1,065 units per milligram and contained at least 90 per cent penicillin G. This material was used in most of the experiments. The other lot was a crystalline sodium penicillin G with a potency of 1,400 units per milligram. Three preparations of penicillin were used: an aqueous solution, a peanut oil suspension, and an emulsion of the aqueous solution in mineral oil. The aqueous solution was prepared by dissolving the penicillin in sterile distilled water. The peanut oil suspension was prepared by mixing a weighed amount of the penicillin powder with the vehicle and shaking before use. The mineral oil emulsion was made by mixing one part of an aqueous solution of penicillin with nine parts of heavy mineral oil and shaking thoroughly before use. The preparations were composed so that a dose of 10 cc. carried the desired amount of penicillin.

The irritating action of the three preparations was tested by injecting them into normal quarters of experimental cows. A physical examination of the udder and tests on milk samples were made at intervals after treatment. The chloride titration, the catalase determination, and the leucocyte count were used to measure the degree of irritation. A few assays were made on the concentration of penicillin in the milk at intervals after injection, employing the cylinder plate method of Schmidt and Moyer.[9] The Breed count was used for leucocyte determinations.

Fifteen dairy herds were selected for a field trial. One of these was an institutional herd of 57 cows which was milked three times daily, while the others were farm herds, each consisting of 13 to 45 animals, milked twice daily. A uniform procedure was followed for all the herds. A physical examination was made of all the cows in the herd, and quarter samples were collected aseptically to detect the quarters infected with streptococci. Then

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From the Department of Veterinary Science, University of Wisconsin, Madison.

The penicillin for these studies was furnished by Chas. Pfizer and Co., Brooklyn, N. Y., and Commercial Solvents Corporation, New York, N. Y.

each infected quarter was treated with penicillin in one of the three carriers. Samples were collected from the treated quarters about ten days after therapy and thereafter at intervals of one week until three successive tests had been made.

Prior to sampling, a stream of milk was discarded into a strip cup. Then the foremilk was collected into sterile screw-cap vials containing a solution of bromcresol purple. Within two hours after collection, the laboratory tests were initiated. The markedly abnormal milk or samples from dry udders were mixed with sterile skimmilk to provide a good growth medium for streptococci. The samples were incubated at 37 C. for sixteen hours as a Hotis test, but when no gross change occurred, they were incubated six hours longer. Smears from each sample were stained with Newman's stain and examined microscopically to determine the morphology of organisms and the relative number of leucocytes. A loopful of gravity cream from the incubated sample was streaked on a blood agar plate containing 0.01 or 0.005 per cent sodium azide. After one day's incubation at 37 C., these plates were examined for colonies of streptococci. In doubtful cases, colonies were transferred into litmus milk for further examination. A few representative cultures of streptococci from each herd were typed by biochemical and serologic means to establish their specific identity.

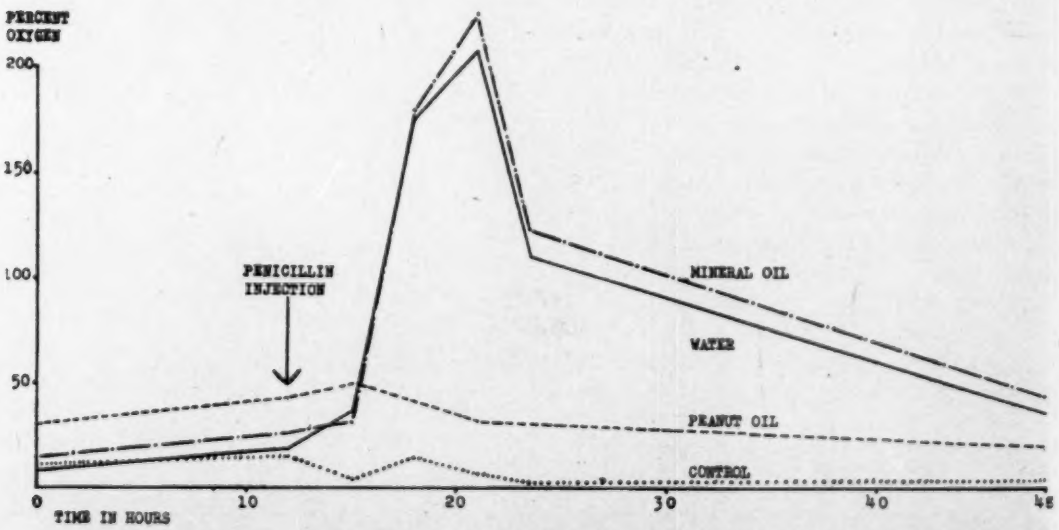
A quarter was considered infected when the streak plating disclosed streptococci which resembled *Streptococcus agalactiae* in colonial morphology, hemolysis, and microscopic appearance. The few quarters in which the samples were positive to the Hotis and microscopic test, but negative on plating, were also considered infected. The results from each of the three bacteriologic tests were usually alike, but those from streak plating were more reliable.

In a trial on nine herds, 224 infected quarters were treated with a single dose of 50,000 units of penicillin in 10 cc. of one of the three carriers. Fourteen infected quarters were treated with mineral oil alone and four quarters were left untreated as controls. In a trial on six herds, 91 infected quarters were treated with three doses, each composed of 15,000 units of penicillin in either water or mineral oil, and 23 control quarters were treated with mineral oil alone. The injections were given at 24-hour intervals.

The infected quarters were grouped as equally as possible according to herd, stage of lactations, and number of infected quarters per cow. All the infected quarters of an individual were treated with the same preparation of penicillin.

A quarter was considered cured when three consecutive weekly tests after treatment were negative for streptococci resembling *Str. agalactiae.*

Cows from part of the herds were classified according to the relative severity of inflammation in the quarters. The criteria used were the leucocyte count, pH as determined by bromcresol purple, and the presence of flakes or clots on the strip cup. The quarters which produced milk that was markedly alkaline or showed clots on the strip cup and contained over 6 million leucocytes per cubic centimeter



Graph 1—Inflammatory reaction produced in normal lactating quarters by 50,000 units of penicillin in an aqueous solution, a peanut oil suspension, or a mineral oil emulsion as determined by the catalase content of the secretion.

were classed as 4+. Those quarters producing milk that was only slightly alkaline or had a normal pH and over 6 million leucocytes were classed as 3+. The quarters which produced milk of normal pH containing between 3 and 6 million leucocytes were classed as 2+. Quarters producing milk that had from 1 to 3 million leucocytes but was otherwise normal were classed as + and the rest of the quarters were classed as —.

RESULTS

A comparison of the irritating effect of the three preparations of penicillin is depicted in graph 1. The catalase content expressed as oxygen percentage of the secretion obtained from the noninfected quarters of a cow treated with the three preparations is shown. Similar changes were found in the chloride and leucocyte content of the secretion, and a repeat experiment in other normal quarters gave identical results. The mineral oil emulsion and the aqueous solution of penicillin were approximately equal in their irritating effects. Either preparation was considerably more irritating than the peanut oil suspension, the latter producing only a slight increase in leucocytes, chloride, and catalase of the secretion.

TABLE 1—Concentration of Penicillin in the Secretion of Normal Quarters at Intervals after Injection of 50,000 Units

Vehicle	3 hr.	6 hr.	9 hr.	11 hr.
Water	1,620	870	70	45
Peanut oil	492	1,000	540	58
Mineral oil	346	560	128	28

The inflammatory reaction to the three preparations was of short duration and caused no appreciable reduction in milk flow. In general, it is believed that the slight reaction resulting from injection is of little clinical significance. Infected quarters which were treated showed no serious reactions, although slight swellings occurred in a few quarters after treatment. Three quarters of 1 cow, however, which were given multiple doses, showed so much swelling that they were eliminated from the experiment after two doses had been given.

The concentrations of penicillin in the milk at intervals after therapy in one experiment are indicated in table 1. There was no marked difference between the three carriers except at the ninth hour. At that time, the quarters injected with the aqueous solution, the peanut oil suspension, and the mineral oil emulsion showed a concentration of 70, 540, and 128 units per cubic centimeter, respectively. At the sixth and the eleventh hour, there were only slight differences between concentrations produced by the three vehicles. While the oils tend to prolong the high concentration of penicillin slightly, the presence of the sustained amount is unlikely to account for the variation in therapeutic efficiency.

The efficacy of the preparations in eliminating the infection is compared in table 2. The data show that the mineral oil emulsion was more efficacious as a carrier for penicillin than were the aqueous solution and the peanut oil suspension. Apparently the peanut oil suspension was inferior to water. The difference between the mineral oil emulsion and the aqueous solution was not marked with the single dose of 50,000 units. With three doses, however, the mineral oil emulsion was much superior to the aqueous carrier. Of the 45 infected quarters treated with three doses of the penicillin-mineral oil emulsion containing 15,000 units each, infection was eliminated in 60 per cent of the quarters. Of the 46 quarters treated with the aqueous solution of penicillin containing an identical amount and subjected to analogous therapy, 33 per cent of the quarters were cured.

A chi-square analysis* of these data gave a corrected value of: $X^2 = 5.81$. This indicated that the probability of such a difference occurring by chance was between 0.05 and 0.01, which is significant.

TABLE 2—The Comparative Efficacy of Peanut Oil, Mineral Oil, and Water as Carriers for Penicillin in the Treatment of Streptococcic Infection

Carrier	No. doses	Amt. penicillin dose	Quar. treated	Quar. cured	% cured
Water	1	50,000	80	25	31
Peanut oil	1	50,000	68	13	19
Mineral oil	1	50,000	77	30	39
Mineral oil	1	None	14	0	0
None	..	None	4	0	0
Water	3	15,000	46	15	33
Mineral oil	3	15,000	45	27	60
Mineral oil	3	None	23	5	22

*Mrs. Dorothy M. Brill performed the statistical analysis.

The infected control quarters, the four untreated, and the 14 injected with one dose of mineral oil remained infected. Of the 23 quarters infused with three successive doses of mineral oil each, five were cured according to the test criteria used.

A comparison of the efficacy of treatment with the degree of inflammation is shown in table 3. On the basis of these data, the degree of inflammation showed little relationship to the effectiveness of penicillin.

TABLE 3—The Importance of the Degree of Inflammation of Lactating Quarters on the Efficacy of Intramammary Therapy with Penicillin

No. doses	Degree of inflammation*	No. quar. treated	No. cured	% cured
1	4+	14	3	21
	3+	6	0	0
	2+	21	5	24
	+	43	10	23
	—	48	12	25
Total		132	30	23
3	4+	26	11	42
	3+	19	10	53
	2+	6	1	17
	+	9	3	33
	—	16	8	50
Total		76	33	43

*See "Materials and Methods" of this paper for key to classification.

Clinical results were consistently more favorable than the bacteriologic findings. The milk became physically normal in all quarters treated with penicillin and usually remained normal for ten days to two weeks. Subsequently abnormal milk with flakes, clots, and high leucocyte count began to appear in a few of the quarters which had not been cured by treatment. There were five recurrences of inflammation due to streptococci in the quarters treated once. Of these recurrences, two had been treated with the aqueous solution, two with the mineral oil emulsion, and one with the peanut oil suspension of penicillin. Only one case recurred with abnormal milk in the 91 quarters given three doses; this was a quarter treated with the aqueous solution.

DISCUSSION

The data presented show a much lower therapeutic efficacy for penicillin than those given in most previous reports. It is believed that this is due to the more sensitive methods used in detecting the organism, especially the enrichment method of culturing. The therapeutic experiences with penicillin indicate that this antibiotic is an inhibitory agent for bacterial growth and that it may reduce the number of organisms in the milk to a very low number. The results obtained in testing for streptococci following therapy are irregular, especially with the Hotis test and the microscopic test. A single negative test following therapy, therefore, is of little value and may be grossly misleading if used as a basis for segregation or for further treatment in the herd.

The apparent recovery of five of the 23 control quarters which were treated with mineral oil alone seems unusual since spontaneous recovery is rare. Three of these five quarters were from a cow that had been dry for a long time. The samples obtained from this cow after treatment were, however, small and probably inadequate for a determination of the presence of infection.

When evaluating the result of therapy, the possibility of reinfection of quarters cured by treatment should not be excluded. In all these experiments, however, the infected quarters in the herds were treated with penicillin so that the likelihood of spreading streptococci was minimal. In addition, precautionary sanitary measures such as the use of a mild chlorine solution on the hands, the teats, and the teat cups were instituted and carried out for all the herds under study. Undoubtedly, these measures were effective in reducing reinfection but did not prevent it completely.

The improvement in the quality of milk following penicillin therapy was striking. The number of quarters showing grossly abnormal milk was greatly reduced by treatment. In a few weeks, however, some of the quarters which remained infected began to produce abnormal milk, as indicated by the reports of the herdsman and a retest of treated quarters. The improved quality of milk is a distinct advantage gained from therapy, but this may induce over-confidence in the results of therapy. Critical bacteriologic findings may detect an infection of quarters showing transient improvement.

The clinical application of research findings with penicillin therapy is a problem not yet completely solved. Experimental results have shown that affected herds can be markedly improved in regard to incidence of streptococcic infection and in regard to the amount of abnormal milk produced by a course of treatment of the infected quarters. Control of streptococcic mastitis can be aided greatly by penicillin treatment, although therapy alone may be of only temporary aid. Accurate diagnosis, careful segregation, and sanitation would appear to be still necessary in the average herd for the preservation of the beneficial results of therapy.

A test of the entire herd using the best diagnostic methods available for the detection of infected quarters should be made. The high incidence of subclinical cases of streptococcic infection makes this diagnostic procedure necessary. If no facilities for bacteriologic testing are available, treatment of cases of streptococcic mastitis would appear to be of only temporary value. The procedure of treating all quarters in the herd in the absence of a testing procedure deserves consideration.

SUMMARY

1) An emulsion of 10 per cent of water in mineral oil was more efficacious than an aqueous solution as a carrier of 50,000 Oxford units of penicillin G, when each was used as a single dose in the intramammary treatment of chronic streptococcic mastitis. A peanut oil suspension of penicillin was inferior to the aqueous solution as a therapeutic agent.

2) Three doses each of 15,000 Oxford units of penicillin G in the mineral oil emulsion were superior to three doses of the aqueous solution.

3) Five of 23 quarters showed recovery after treatment with multiple doses of mineral oil alone.

4) The efficacy of treatment was not influenced by the degree of inflammation initially present in the affected quarters.

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Leucemic Lymphoblastoma in a Cow with Involvement of the Udder

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A MATURE Guernsey cow was presented for examination a few days after the development of an extreme distention of the mammary gland and an edema of the ventral thoracic and abdominal regions (fig. 1). The cow had calved normally three months prior to the onset of this illness. The milk flow had decreased gradually from 40 lb. (18.1 kg.) per day to 1 or 2 lb. (0.5 or 0.9 kg.). The appetite, which was not affected at first, gradually diminished until inappetence became pronounced. The local veterinarian was called to treat what appeared to be a case of mastitis. On examination, he found the teats and gland to be distended and firm, but the heat which usually accompanies an acute inflammatory process was lacking. There was no rise of body temperature. The animal appeared dehydrated and the hair was roughened. A rectal examination revealed several irregular tumors involving the genital tract.

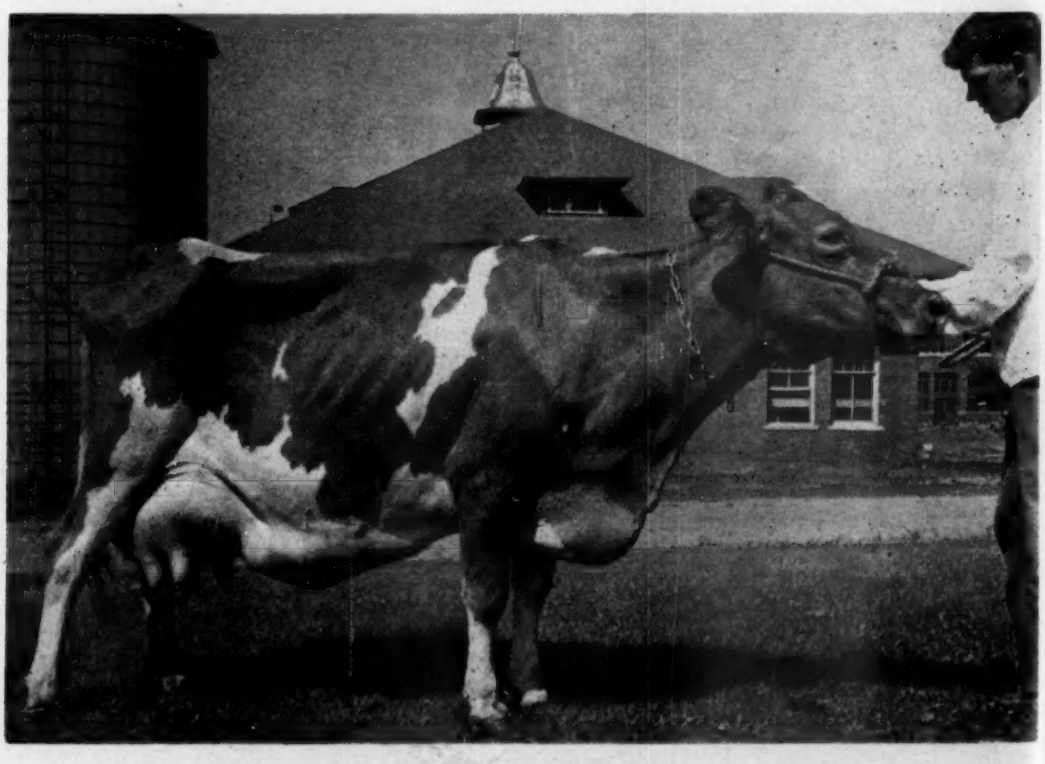

Fig. 1—Appearance of the patient shortly after arrival at the University Farm.

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Chief, Division of Veterinary Medicine, University of Minnesota (Boyd); associate in experimental pathology, Mayo Foundation, Rochester, Minn. (Karlson); assistant (Jasper), assistant (Sellers), and instructor (Collier), Division of Veterinary Medicine, University of Minnesota.

*We are indebted to Dr. Otto I. Kinneberg for the opportunity of studying this case.

When the animal was examined by rectal exploration at the laboratory, it was found that the internal iliac lymph nodes were enlarged. The presence of nodular masses in the genital tract was confirmed. A tentative diagnosis of leucemia was made at that

time. The acute distention of the mammary gland was attributed to interference with the lymph flow from the udder through the supramammary lymph nodes.

The animal was kept for two weeks for further observation. During this period there was no significant variation of the rectal temperature. There was a progressive increase of respiratory rate to 50 or 60 shallow respirations per minute. Abnormal heart sounds were suspected but these were never very definite.

The teats were too tense to permit manual removal of milk, but a total of about 1 qt. (946 cc.) could be obtained by the use of a cannula. The secretion was free of clots, thick, and resembled colostrum. The cell count ranged from 1,700,000 to 5,000,000 per cubic centimeter from the various quarters. A mixed flora consisting of micrococci and various rod-shaped organisms was cultured from each quarter, but none of the microörganisms commonly associated with mastitis were identified.

During the two-week period of observation, the subcutaneous edema of the ventral thoracic and abdominal regions became progressively more extensive. The skin over the ribs became tense. The feces assumed the character of heavy dough, although the animal continued to eat small amounts of green alfalfa hay. There was no detectable alteration of rumen function or intestinal peristalsis. During the last week of observation, the cow was recumbent much of the time. The swelling of the brisket and subcutaneous edema became fluctuant, and the udder was less firm and pitted on pressure.

An aspiration biopsy of the liver revealed a small focus of lymphocytic infiltration.

Blood counts showed marked leucocytosis, due largely to an increase in the number of cells reported as lymphocytes, as indicated in table 1.

About 60 per cent of the lymphoid cells were unlike those seen in normal cow's blood. They varied in size up to twice the

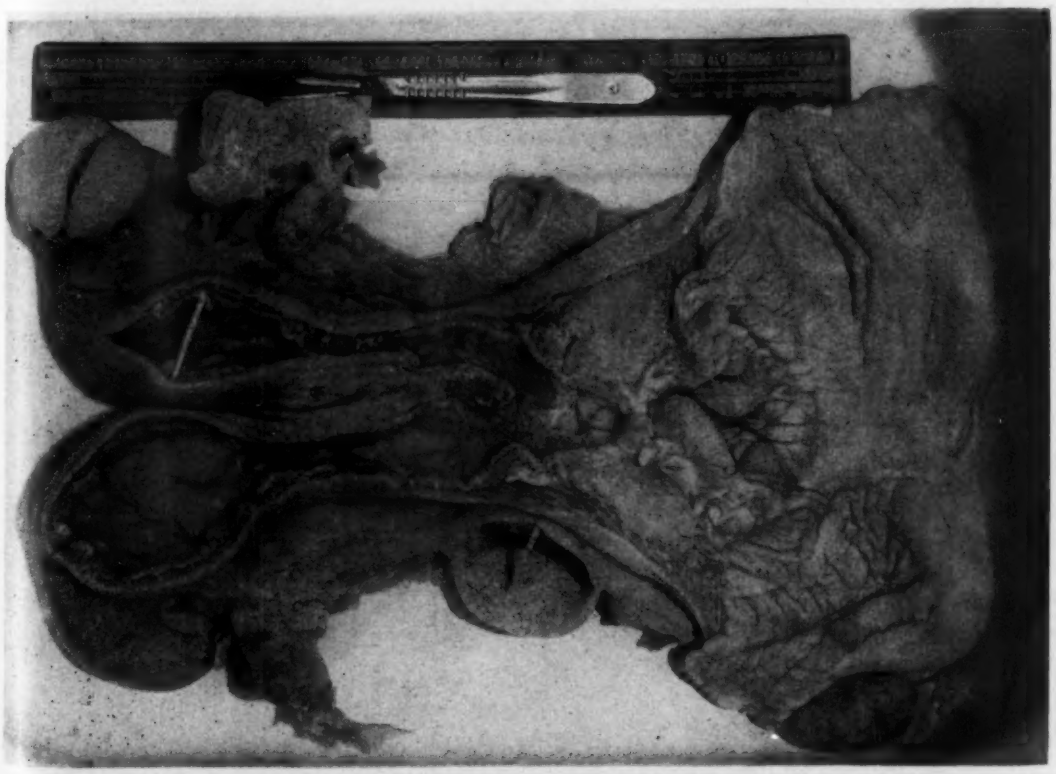

Fig. 2—Genital tract showing tumor masses.

diameter of the average lymphocyte. The scanty, faintly basophilic cytoplasm had a spongy appearance, and a few cells contained indistinct acidophilic granules and rods. The large nucleus varied in shape from round to trilobed. A few of the large cells had a fine granular appearance of the chromatin with no clumping of nuclear material, but in most cells the chromatin was arranged in fine strands connecting distinct rectangular blocks. Pale blue, oval nucleoli were evident in all the cells that had granular chromatin but were seen in only about 30 per cent of the other types.

TABLE 1—Hematologic Data*

1946	Erythrocytes /cmm. Blood	Leukocytes /cmm. Blood	Differential Count		
			Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
Aug. 1	4,560,000	15,800	76	19	5
Aug. 5	6,010,000	23,200	94	5	1
Aug. 6	5,320,000	21,050	95	5	0
Aug. 7	6,330,000	26,100	..	..	..
Aug. 8	5,480,000	20,900	91	8	1
Aug. 9	6,060,000	28,100	89	11	0

*Concentration of hemoglobin on August 1: 12.3 Gm. per 100 cc. of blood.

The cow was destroyed for humane reasons after two weeks of observation. Besides selecting tissues for histologic examination, tissue imprints were made from various lymph nodes, spleen, bone marrow, and organs that had macroscopic evidence of involvement by the neoplastic process.

Postmortem Report

Lymph Nodes. — The supramammary lymph nodes were greatly enlarged. The left node measured 26 by 16 by 10 cm. On cross sectioning, it was found to contain a large cistern filled with old clotted blood around the periphery with a lesser amount of fluid blood centrally. The right supramammary node was somewhat smaller, measuring 20 by 17 by 8 cm. It was solid in consistency throughout but showed some hemorrhage into the central tissues. The internal iliac lymph nodes were somewhat enlarged. On sectioning, they appeared pinkish gray, the cut surface bulged, and the tissues seemed more friable than normal. The left node measured 15 by 10 by 4 cm. and the right, 10 by 9 by 4 cm. A num-

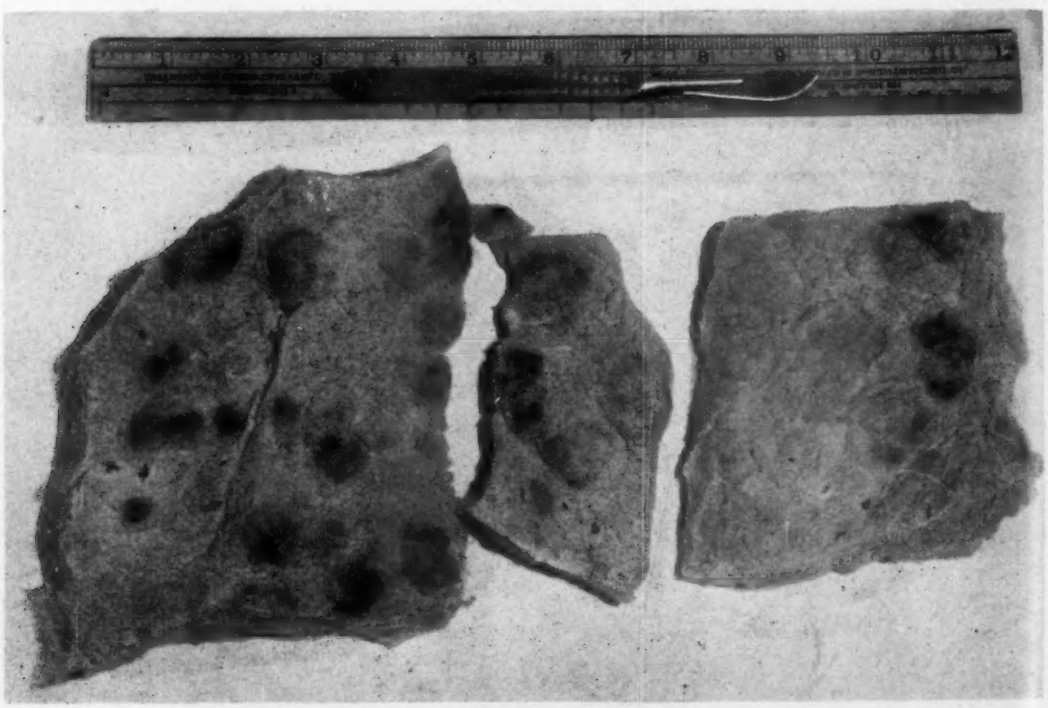

Fig. 3—Cross sections of mammary tissue showing scattered nodules of neoplastic tissue.

ber of petechial hemorrhages were noted on the cross section of the right node. The left prefemoral lymph node measured 13 by 5 by 2 cm. and was normal in appearance. The right precrural lymph node was slightly smaller and also normal in appearance. The left external iliac lymph node appeared slightly enlarged and softer than normal. It measured 6 by 3 by 2.5 cm. Petechial hemorrhages on cross section were numerous. The right external iliac lymph node showed similar involvement to a lesser degree. The posterior mediastinal lymph nodes consisted of four nodes which were enlarged and flabby. They varied in length from 2 to 7 cm. In each case, they presented, on cross section, a region of apparently normal tissue surrounded by a "rind" of new growth. The left prescapular lymph node measured 13 by 4.5 by 2 cm. It appeared somewhat enlarged and flabby. However, the cut section appeared normal. The visceral lymph nodes and the regional nodes not already discussed were examined and appeared to be normal.

Genital Tract (Fig. 2).—The left ovary contained a corpus luteum 2.5 by 1.5 cm. The left mesovarium contained a nodule, roughly circular and about 3 cm. in diameter, near the attachment to the parietal wall. The right ovary was normal in size. It contained a follicle 0.7 cm. in diameter. The tissue between the horns of the uterus beyond the bifurcation contained several small nodules ranging from 1 to 3 cm. in diameter. A nodule roughly 5 cm. in diameter was found near the tip of the right horn involving the uterine wall. Two distinct nodules were located in the region of the cervix. One nodule lay to the left of the median line and measured 5 by 4.5 by 3 cm. The second nodule lay in the tissues of the uterine end of the cervical canal and measured 4.5 by 2.5 by 3 cm. When incised, the lesion in the right horn was found to be fairly hard in consistency, and the cut surface appeared as a homogeneous, light yellow substance with a bulging surface. There were no regions of infiltration in the body of the uterus. On incision of the left uterine horn, two distinct regions of infiltration of the mucosa were apparent, near the bifurcation and near the middle of the horn, respectively, measuring about 2 and 5 cm. in diameter. The regions were raised and appeared yellow beneath the mucosa covering them.

Several folds of the cervical canal were enlarged and firm. The larger folds measured 2.5 by 2 by 3 cm. The cut surface of these enlarged folds was a homogeneous, glistening yellow. The nodule previously described as involving the cervical canal at the uterine end was a yellow mass containing somewhat more fibrotic tissue than the other nodules described. The nodule to the left of the cervix showed a homogeneous, yellow, glistening cut surface. On incision of the vagina, four regions of infiltration of the mucosa were apparent. These all consisted of nodular, raised, yellowish areas, well-circumscribed, varying from 1 to 4 cm. in diameter.

Mammary Gland (Fig. 3).—The parenchyma contained numerous scattered nodular lesions varying from 0.5 to 4.0 cm. in diameter on cross section. Some of these lesions were nearly colorless, but most of them were hemorrhagic. These infiltrated regions were not well separated from the mammary tissue on gross appearance. The distribution of the nodular lesions appeared to be about equal in all four quarters. The lesions were more numerous near the base of the udder than in the lower part of the udder near the teat cisterns. There were 12 nodules in one area 15 cm. square near the base of the udder.

Heart.—The pericardium and the pericardial fluid appeared normal. The wall of the left auricle was involved with a light yellowish, elongated nodule 8 by 4.5 by 1.5 cm. A smaller nodule 1.5 cm. in diameter was palpable at the junction of the auricle and ventricle. A nodule measuring 3 by 2 cm. was visible on the epicardial surface of the right auricle. There was a light yellow, elevated area 1.5 by 1 cm. on the epicardial surface of the right ventricle. Viewed from the endocardial surface, the lesion in the right auricle appeared as a great thickening of the muscular wall. In the posterior wall of the right auricle, a lesion 5 by 4 by 2.5 cm., not visible from the external surface,

was noted. This lesion was raised, yellow, and sharply defined. The small lesion noted on the epicardial surface of the right ventricle did not extend through the wall but involved the outer layer of muscle. Numerous, small infiltrated regions were noted beneath the endocardium. One nodular lesion, 1 cm. square, was found deeply embedded in the wall of the left ventricle.

Lungs.—There was a region of peribronchial infiltration starting at the bifurcation of the trachea and surrounding the right bronchus. This mass measured 14 by 8 cm. on the cut surface. The infiltrated tissue surrounded a lymph node 3.5 cm. in diameter.

Liver.—There were about 12 subdiaphragmatic nodules on the liver about 0.5 to 3.0 cm. in diameter. These nodules were just beneath the capsule and did not appear to invade the hepatic tissue. There was an irregular region of infiltration beneath the capsule on the diaphragmatic surface near the posterior margin of the liver but not invading it.

Kidneys.—There were eight subcapsular nodules in one kidney and 12 in the other. These lesions varied from 1.0 to 1.25 cm. in diameter and extended into the cortex.

Spleen.—The spleen measured 55 by 15 by 4 cm. and appeared normal, except that the follicles appeared somewhat prominent on cross section.

Gastrointestinal Tract.—A few scattered nodules averaging 1 cm. in diameter were observed on the serosa of the rumen and abomasum. There were about 15 round soft nodules up to 2 cm. in diameter in the mesentery of the small intestine, not associated with the mesenteric lymph nodes. (The mesenteric lymph nodes were grossly normal.) There were six similar nodules in the wall of the lower part of the jejunum. The largest of these lesions measured 9 by 3.5 by 2.5 cm. The apex of the cecum showed a large hemorrhagic region of the subserosa measuring 10 cm. in diameter. An infiltrated nodular lesion measuring 1.5 by 1.0 by 0.5 cm. was observed in the wall of the colon.

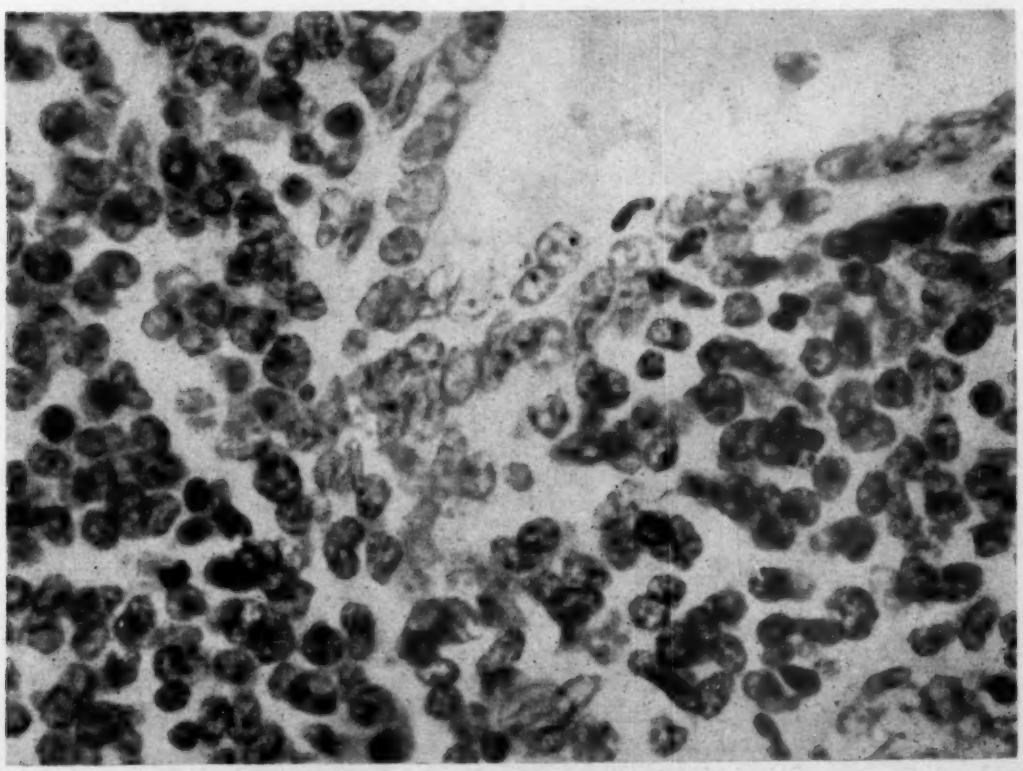

Fig. 4—Mammary gland. Note pleomorphism of nuclei and cytoplasm. x 800.

Other Lesions.—Just posterior to the xiphoid cartilage, there were extensive adhesions involving the diaphragm, the greater omentum, and the abdominal wall. Within the fibrous adhesion tissue, there was a hard, irregular mass measuring 14 by 10 by 8 cm. Around the periphery of this mass, but separated from it, were several smaller spherical nodules varying from 1 to 4 cm. in diameter. On the adjacent part of the diaphragm near the reflection onto the abdominal wall, a white area about 1 cm. in diameter was visible. On palpation, it appeared to be a flat plaque.

Microscopic Examination.—The neoplasm in all situations was composed of large, rounded cells up to twice the diameter of a normal lymphocyte seen in formalin-fixed tissue sections. Some adjoining cells appeared to be connected by strands of cytoplasm, but there was no syncytial arrangement and in general the cells were discrete. The narrow rim of basophilic cytoplasm formed short, blunt projections in some cells, and in others it was round or conformed to the shape of the nucleus, which was round, oval, or kidney shaped (fig. 4). The vesicular nucleus, several times larger than that of a normal lymphocyte, had a fine distinct border, and the chromatin was arranged in fine strands and granules. Many cells in different phases of mitosis could be seen.

Sections of lymph nodes revealed a complete loss of normal structure. In the case of lymph nodes visibly involved at the postmortem examination, there was a uniform tissue composed of the large cells with no distinction between medullary and cortical portions, no follicles, and no sinuses. The capsule was invaded by the neoplastic tissue. No normal lymphocytes were evident anywhere. In the lymph nodes, such as the mesenteric, that grossly had little or no evidence of involvement, there was also a marked alteration of the normal pattern, but there were remnants of follicles that appeared to be compressed by the advancing neoplastic tissue. In several lymph nodes, the follicles were represented by thin

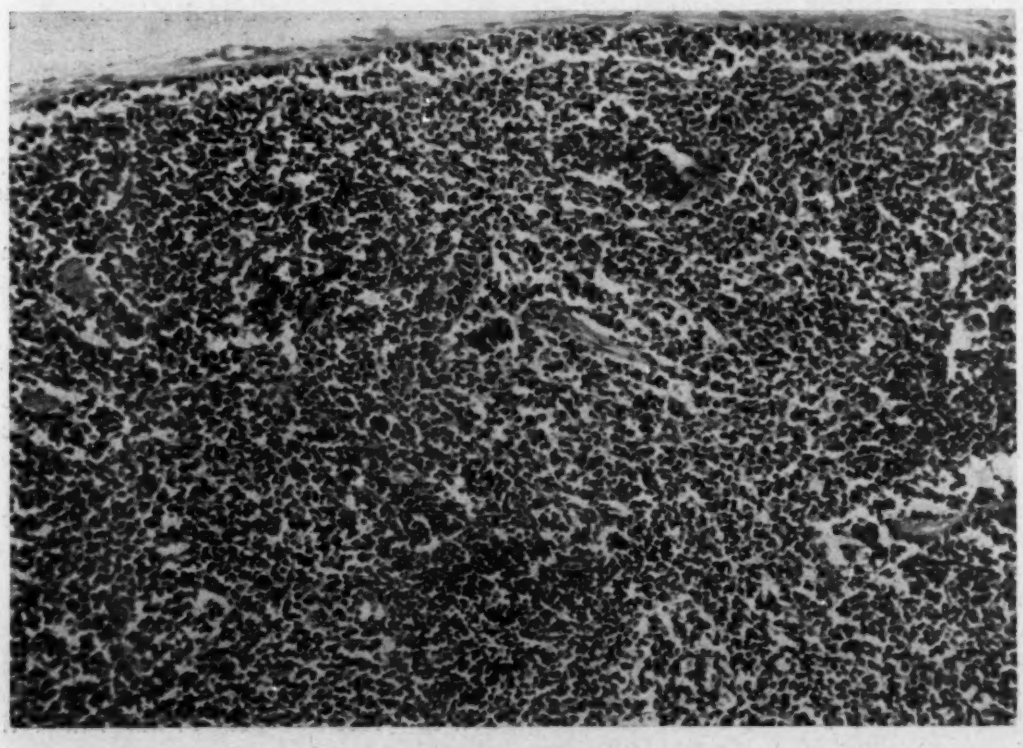

Fig. 5—Mesenteric lymph node showing marked alteration of normal pattern. x 140.

zones of normal lymphocytes surrounding a large germinal center composed of the large new cells. Many mitotic figures were seen here. Peripheral sinuses could be recognized as channels filled with free rounded cells identical morphologically to those forming the various tumors (fig. 5).

The spleen appeared to be diffusely involved. In one section, the pulp was entirely replaced by a uniform distribution of the neoplastic tissue, and the organ could be recognized only by the trabeculae and a few remnants of follicles which were thin rims of normal lymphocytes surrounding a mass of neoplastic cells. Another section of spleen revealed a few distinguishable splenic sinuses distended by a mixture of erythrocytes and free tumor cells. This section had many reticulum cells filled with brown pigment.

There were portal infiltrations of the large lymphoid cells in the liver from which the neoplastic process extended into the lobules by following along the cords of hepatic cells. A section through a nodule in the serosa of the small intestine showed invasion through the muscle coats up into the mucosa. The epithelium had been replaced, forming an ulcer which was not detected grossly. The kidneys showed infiltration of the cortex and invasion through the overlying capsule. There was no evidence of invasion into tubules.

The grossly normal node in the right lung, surrounded by tumor tissue, had a microscopic appearance similar to that described for the mesenteric lymph nodes except that no definite follicles could be seen. The sinuses were filled with free rounded cells resembling those of the node and the surrounding new growth. Adjacent alveoli were compressed. The myocardium showed separation of the muscle fibers by infiltrating neoplastic tissue which appeared to extend out through the epicardial fat. In one place, there was a polypoid growth extending into the lumen of a large, thin-walled vessel and pushing the intact endothelium ahead of it.

The mammary gland (fig. 4) had broad, irregular regions of neoplastic tissue surrounding and separating alveoli and ducts. There was no evidence that the epithelial lining of the glandular structures was being invaded.

Impressions of bone marrow from the fourth and seventh ribs of the left side showed no evidence of involvement there. Impressions of lymph nodes presented a much greater variation of cell types than was seen in tissue sections. This was especially true for those lymph nodes that had remnants of follicles and scattered normal appearing lymphocytes, as seen in the mesenteric nodes. In these, one could see large and small lymphocytes with a narrow basophilic cytoplasm and dense chromatin pattern resembling the predominant cell type in impressions of normal bovine lymph nodes. However, the majority of cells in all the lymph nodes were quite unlike those of the normal tissue. They were several times the diameter of the usual lymphocyte and had an almost invisible basophilic cytoplasm. The nucleus was spherical in most of these cells, but oval and indented shapes were seen. The chromatin was arranged in fine strands and rectangular blocks. Some nuclei were formed of fine chromatin granules. Such cells as these may be found in impressions of normal bovine lymph nodes, but in normal nodes they are present in small numbers. Mitotic figures were readily seen and most of the cells had two to four nucleoli.

Impressions of the enlarged supramammary lymph nodes revealed very few normal appearing lymphocytes, a few plasma cells, and some neutrophils mixed in with the predominating large, typical lymphoid cells. Impressions of the neoplastic tissue in myocardium, liver, kidneys, lungs, and intestine presented cells identical to the predominant cells of the lymph nodes. The atypical cells of blood films were morphologically identical to those seen in tissue smears.

COMMENT

Examination of the lymph nodes that retained some normal structure suggested that the process was one in which the reticulum had become neoplastic and was form-

ing the large lymphoid cells that appeared in the blood and composed the nodules and infiltrations in various organs. This was suggested by the appearance in lymph nodes where the lymph follicles were being compressed by the widening germinal centers. The reticulum itself, rather than the lymphocytes, appeared to be active. This appearance in widely separated lymph nodes suggested that the process might be multicentric and that the neoplasm in various lymph nodes arose from the reticulum there instead of being metastatic. The presence of the new growth in organs may be explained on the basis of metastasis or extension from adjacent lymph nodes or possibly development from preëxisting reticulum cells.

Involvement of the mammary gland is rare in this disease of cattle. We have not seen it in 15 cases previously observed. Franke[5] reported that it was not observed in the large number of cases he had seen in a packing plant. Junack[6] stated he had seen only 1 cow with this tumor in which the udder was involved.

The confusing terminology of these diseases makes it difficult to arrive at a designation acceptable to all. The disease is not the lymphosarcoma originally described by Kundrat[2] but resembles leucosarcoma, described by Sternberg[4] and more recently proposed by Moon.[3] This latter designation appears to be indefinite in that it does not indicate the cell type but merely emphasizes the secondary blood changes or leucemic state. The term "lymphocytoma" seems inadvisable, since it suggests that the cells are lymphocytes, which they clearly are not. They may resemble lymphoblasts, and the term "leucemic lymphoblastoma" is used in the present paper.

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The Organisms Causing Bovine Mastitis in Puerto Rico

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MASTITIS is common among dairy cattle in Puerto Rico and the cause of great economic loss. Believing it would be desirable to know which organisms are producing mastitis on this island, a bacteriologic study was made of milk samples and purulent discharge from affected cows.

METHODS

Collection of Samples.—Udders were thoroughly washed with tap water, then with chlorinated water and dried with a clean hand towel. In many instances, the teat opening was also wetted with 70 per cent alcohol. Samples were taken after the teat was dry. Approximately 1 cc. of the foremilk was discarded and amounts ranging from 5 to 15 cc. were collected into sterile test tubes 150 by 10 mm. In the majority of cases, individual samples were taken from each quarter. In other instances, composite samples from the four quarters were collected into a single tube. The tubes were placed in ice immediately after collection and brought to the laboratory where they were cultured immediately or placed in the ice box for one or two hours, after which cultures were made.

Culturing of Specimens.—Beef-heart infusion agar (Difco), to which 3 per cent horse blood was added, was used. The milk in the tube was mixed with the aid of a sterile 1 cc. pipette, 0.1 cc. placed on the surface of the agar and spread over the surface of the medium with a sterile cotton swab.

When purulent material was to be cultivated, a Gram stain was made as a preliminary step and the size of the inoculum was adjusted accordingly.

Examination of cultures.—The plates were incubated aërobically at 37 C. and examined after twenty-four, forty-eight, and seventy-two hours. Each type of colony was studied morphologically. The streptococci were identified by their biologic characteristics and by grouping the strains by Lancefield's method.

RESULTS

Streptococcus agalactiae is undoubtedly the cause of the vast majority of cases of bovine mastitis in this island (table 1).

Corynebacterium pyogenes was obtained in pure culture from 26 per cent of the animals examined in herd 3. Hemolytic *Staphylococcus albus* was cultured in large numbers from 4 cases. The five strains designated as unclassified streptococci did not belong to any of Lancefield's groups. Their biologic properties were not studied.

Many colonies of *Str. agalactiae* and *Sta. albus* were also cultivated in small numbers from five milk samples from different animals. In one instance, many colonies of *Sta. albus* were present, together with numerous colonies of *Str. agalactiae.*

The milk of 12 cows from herd 3 with no clinical signs of mastitis and giving apparently normal milk gave the following results when cultured:

No. of animals studied	12
No. yielding *Str. agalactiae* in varying numbers	3
No. yielding a few colonies of *Sta. albus* only	4
No. yielding a few colonies of *C. pyogenes*	1
No. yielding a few colonies of colon bacilli	1
No. yielding a few colonies of diphtheroid bacilli	1
No. yielding no growth	2

In herd 4, 46 of the 50 cows examined were shedding *Str. agalactiae* in large or moderate numbers. In 32 instances, the Streptococcus growths were mixed with hemolytic or nonhemolytic *Sta. albus* (in 13 cases, a few colonies; in 9 cases, some colonies; in 9 cases, moderate numbers of colonies; and in 1 case, numerous colonies). In 2 cases, a very few colonies of colon-like bacilli were mixed with streptococci and staphylococci. Two plates showed subtilis contaminations and from 9 samples chromogenic (various grades of yellow) gram-positive cocci were cultivated besides *Str. agalactiae* and *Sta. albus.*

In herd 5, *Str. agalactiae* was recovered from 45 of the 47 cows examined. In 15 cases, hemolytic and nonhemolytic *Sta*

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albus was also present (2 cases had numerous colonies and 13 cases had a moderate number of colonies).

In 1942, 27 cows with clinical mastitis from herd 2 were studied bacteriologically with the results shown in table 1. In 1946, all the lactating cows in this herd, irrespective of clinical mastitis, were studied. Cultures were made of the milk from 265 animals. *Streptococcus agalactiae* (Lancefield's group B) was recovered from 162 cows (61%). From 12 cases, streptococci not belonging to any of Lancefield's groups were isolated. In the vast majority of cases, these agalactiae streptococci were recovered from cows with clinical mastitis. However, *Str. agalactiae* was occasionally recovered from apparently normal udders and in milk from cows in which the infection was not suspected at the time the samples were taken. The 12 strains of streptococci not belonging to Lancefield's group B gave the biological reactions characteristic of *Streptococcus uberis*. We can not state definitely that these organisms were associated with clinical mastitis.

Hemolytic or nonhemolytic *Sta. albus* was cultured from one or more quarters of 102 cows. In 41 cases there were from one to ten colonies on the plate; in 27 cases from ten to 25 colonies; in 18 cases from 25 to 75 colonies; and in 16 cases more than 75 colonies.

In 8 cases, in which more than 75 *Sta. albus* colonies were obtained on the plate, this was the only organism recovered. However, in all instances, except one, the milk from which the organism was recovered was macroscopically normal and there was no clinical evidence of mastitis. The single exception was a cow with clinical mastitis giving bad milk from which a pure and abundant growth of hemolytic *Sta. albus* was the only organism recovered in three attempts at four-day intervals. In 52 instances, *Sta. albus* was found on plates on which *Str. agalactiae* was the predominating organism.

From 80 of the animals examined, hemolytic or nonhemolytic *Staphylococcus aureus* was recovered. In the great majority of cases, only from one to five colonies were present on the plates. Only in three instances did more than 50 colonies develop from any sample. The milk samples in these cases were macroscopically normal and came from cows with no clinical evidence of mastitis.

Thirteen strains of *Sta. albus* and seven of *Sta. aureus* were tested for the produc-

TABLE 1—Organisms Isolated in Puerto Rico from Milk or Purulent Material from Cows with Clinical Mastitis

Herd No.	No. Cows Examined	No. and % pos.	Organisms predominating or found in pure culture						
			Str. agalactiae	Unidentified streptococci	*C. pyogenes*	*Sta. albus*	*B. pyocyaneus*	*B. coli*	No growth
1	12	No. pos.	12	0	0	0	0	0	0
		% pos.	100	0	0	0	0	0	0
2	27	No. pos.	26	0	0	1	0	0	0
		% pos.	96.3	0	0	3.7	0	0	0
3	27	No. pos.	16	0	7	2	0	1	1
		% pos.	59.7	0	25.6	7.3	0	3.7	3.7
4	50	No. pos.	46	3	0	1	0	0	0
		% pos.	92	6	0	2	0	0	0
5	47	No. pos.	45	2	0	0	0	0	0
		% pos.	95.7	4.3	0	0	0	0	0
Other herds	27	No. pos.	20	0	4	0	1	2 Hemolytic	0
		% pos.	74	0	15	0	3.7	7.3	0
Totals	190	No. pos.	165	5	11	4	1	3	1
		% pos.	87	2.6	5.8	2.1	0.5	1.5	0.5

tion of coagulase using human plasma with negative results.

We think the *Sta. albus* strains were mostly contaminants or transitory inhabitants of the teat canal of these cows. Diphtheroid bacilli, gram-positive micrococci, sarcina, *Bacillus subtilis*, and colon bacilli were the contaminants commonly found.

SUMMARY AND CONCLUSIONS

Streptococcus agalactiae (Lancefield's group B) is the organism mostly concerned with the production of bovine mastitis in Puerto Rico. This organism has been isolated either in pure culture or has predominated in mixed cultures with *Staphylococcus albus* in 87 per cent of the cases of clinical mastitis studied. This organism has also been isolated occasionally from the macroscopically normal milk of cows with no evidence of clinical mastitis.

Corynebacterium pyogenes was responsible for the mastitis in 5.8 per cent of the cases.

Hemolytic and nonhemolytic *Sta. albus* was very commonly present. However, it was obtained in pure culture or predominated in only 2.1 per cent of the animals with mastitis examined. In one instance, it was obtained repeatedly in large numbers and in pure culture from a case of mastitis. *Staphylococcus aureus* was frequently present but in very sparse numbers. *Escherichia coli* was cultivated in large numbers from 3 cases.

Isolation of Clostridium Hemolyticum from Bones

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DURING the course of investigations on bacillary hemoglobinuria (red water disease) in Montana, there have been instances in which the disease has been known to spread to adjoining ranches from sources other than natural drainage, and the accidental intermingling of infected with noninfected animals. The possibility of coyotes and crows acting as vectors, by the spreading of tissues from improperly destroyed carcasses, has been suspected as a source of the disease for some time. In one instance, a diagnosis of red water was made in a cow that had been seen chewing on a bone a few days before the animal developed symptoms of the disease. In this case the bone had been carried from some distance to the clean premises by a dog.

As a result, bone specimens from 12 carcasses found in the red water area from time to time have been collected and examined bacteriologically for the presence of *Clostridium hemolyticum*.

EXPERIMENTAL PROCEDURE

A slight modification of the usual anaërobic procedures was found to be of value in the isolation of *Cl. hemolyticum* from some of these contaminated specimens.

It was reported in a previous article[1] that when the toxin of *Cl. hemolyticum* is combined with an egg-yolk emulsion (lecithovitellin), opalescence and flocculation occur. The reaction has been attributed to the enzyme lecithinase, which is present in this toxin. An apparent parallelism exists between the lecithinase and hemolytic and lethal activities of this toxin. Although similar reactions occur in this medium with the toxins of *Clostridium welchii* type A, *Clostridium botulinum* type A, and *Clostridium oedematiens*, the reaction in each case is specifically inhibited by the antiserum of the toxin causing the reaction.

By titrating the supernatant fluid of centrifuged cultures from these bones in a red blood cell suspension for hemolytic activity, and in a lecithovitellin solution for lecithinase activity, and by further testing positively reacting culture fluids for inhibition of these activities in the presence of bacillary hemoglobinuria antiserum, it was possible to determine the presence or absence of *Cl. hemolyticum* in such contaminated cultures.

In general the procedure used was as follows:

Original inoculations of the moist or dry bone marrow were made into peptic digest medium adjusted to give a pH of 7.4 after sterilization. These cultures were heated to 80 C. for twenty minutes before incubation. After incubation for sixteen to eighteen hours at 37.5 C. the supernatant from the centrifuged cultures was titrated by adding descending quantities to (1) a 1.5 per cent suspension of rabbit red blood cells in physiologic saline and (2) a lecithovitellin solution (preparation previously described).[2] At the same time, control tubes containing 0.1 cc. of the supernatant and 0.1 cc. of bacillary hemoglobinuria antiserum were combined with the above indicators. Titration readings were made after 6 hours' incubation at 37.5 C. Only those cultures showing complete hemolysis in a red blood cell suspension and flocculation in the lecithovitellin solution at dilutions of 1:50 or higher, and a complete inhibition of both activities in the presence of *Cl. hemolyticum* antiserum, were carried into 1 per cent deep agar shakes for further purification. Subsequent cultures made from picked colonies from the deep agar shakes were also checked for their lecithinase and hemolytic activity. It was sometimes necessary to make several passages through deep medium before pure cultures were obtained. When cultures were apparently pure, their toxicity was determined by intravenous inoculations of the supernatant into Swiss mice. Specificity of strains isolated was checked by toxin-antitoxin neutralization tests in mice, using the clarified supernatant as the toxin and University of Nevada bacillary hemoglobinuria antiserum as the antitoxin. A further check on the specificity of the

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strains was made by preparing antigens and determining their agglutinability against *Cl. hemolyticum* antiserum.

Of the 12 specimens examined, three were bones from animals dead of an unknown cause; seven were bones from suspected red water cases, and the remaining two were bones from animals known to have died of red water disease. One of these latter specimens was a partially burned bone from an improperly destroyed carcass. All of the bones were from animals that had been dead six months to a year.

Isolations of *Cl. hemolyticum* were made from bones of three of these carcasses. One strain was recovered from the partially burned bone, and the other two were recovered from bones of the suspected red water cases. Bones from carcasses in which isolations were made were placed in a special container on the laboratory roof and exposed to the weather for one year. At the end of the year, isolations of *Cl. hemolyticum* were again made from these specimens.

DISCUSSION

The recovery of *Clostridium hemolyticum* from bones of animals dead of red water disease after one to two years is not surprising in view of the protected location of the organisms within the bone marrow cavity and since the organism is known to be one of the spore forming anaërobes. The failure to recover *Cl. hemolyticum* from the bones in 1 known positive and several suspicious cases of bacillary hemoglobinuria may be explained on the premise that probably all animals dying of this disease do not develop a generalized blood stream infection before death. The fact that *Cl. hemolyticum* can be recovered from bones two years after death of an animal is sufficient evidence that an improperly destroyed or lost carcass is a potential reservoir of future infections.

Trichomonas Foetus Infection and Bovine Reproduction

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ALTHOUGH bovine venereal trichomoniasis has been recognized as a distinct entity for more than fifteen years, considerable confusion still exists as to what constitutes the typical clinical picture of a herd affected with this disease. Early authors[1,2,3] were impressed by the occurrence of abortion and pyometra and considered these to be the dominant symptoms of *Trichomonas foetus* infection. A few later authors considered the malady as an abortion disease and termed it trichomonad abortion. Fulminating postcoital vaginitis was described by certain authors.[3,4] To others,[3,5] a severe "raspy" vaginitis during infection was reported as characteristic. Several workers stated that the course of this disease may differ in herds or localities.

The data or observations of recent workers[6,7,8] have failed to confirm certain of the earlier descriptions of *T. foetus* infection, particularly in regard to gross pathology, and relative incidence, and the importance, in diagnosis, of abortion of recognizable embryos and fetuses, and of pyometra.

Some of the confusing and conflicting concepts of bovine venereal trichomoniasis arise from the fact that many reports have been based on observations of infected herds whose reproductive histories were incomplete—studies which have not been extended over sufficiently long periods to encompass the complete course of infection in the individual female. Thus, readily apparent and dramatic incidents tended to rise above their actual importance. Since prolonged observations under controlled conditions are seldom possible in a commercial herd, this is understandable.

To provide cattle that could be observed constantly over a period of years, a herd was established in 1936 by the Zoölogical Division, U. S. Bureau of Animal Industry, at the Agricultural Research Center, Beltsville, Md. It has been continuously maintained subsequently for the exclusive purpose of studying bovine venereal trichomoniasis.

It is the purpose of this paper to present, in considerable detail, specific experimental data relative to the reproductive performances and *T. foetus* infections of females in this herd and, also, to discuss these data as they relate to certain practical aspects of trichomoniasis control. In so doing, it is hoped that some of the reasons for the variety of symptoms reported, resulting from infection with this pathogen, will become evident, and that the illusion of capriciousness of the course of *T. foetus* infections, when seen in the light of these long-time studies, will be resolved into a lucid rational picture.

Materials and Methods

As these experiments were in progress for nine years, several changes in the personnel collecting these data occurred. From September, 1936, until May, 1941, the work was done by Charles W. Rees and/or George Garlick; from May, 1941, until August, 1941, by John F. Christensen; from the latter date until the end of 1942 by Datus M. Hammond and/or the present writer; and through 1943 until the present time by the present writer. In the tables and text, the numbers designating experimental animals studied entirely, or in part, by Rees and/or Garlick are followed by an R.

The 29 heifers and cows, the case histories of which are reported, were grades or of mixed breeding. During the summer months, they were kept on pasture or in dry lot; during the winter, they were housed in a dairy barn at night and kept in an exercise lot during the day. Their ration consisted of ample quantities of good quality legume hay supplemented by grain, and pasture when available. Periodic tests for brucellosis and tuberculosis at no time revealed infected animals.

Precautions were taken to preclude the in-

This work was carried out at the United States Department of Agriculture, Agricultural Research Center, Beltsville, Md.

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troduction of actively infected or previously infected heifers into the herd. The females purchased from outside herds were sexually immature virgins from trichomonad-free sources. All heifers in the herd were examined weekly from puberty until their first experimental exposure to detect any nonvenereally transmitted infection.

Rees and Garlick occasionally varied the frequency of their sampling. Usually, however, samples were examined daily for the first few months subsequent to exposure, then twice weekly. In the latter part of these experiments, with but few exceptions, specimens were examined either five or six times each week.

Conditions for the observation of signs of estrus by Rees and Garlick were not advantageous. Their data dealing with the occurrence of estrus have not been used in this paper. However, absence of estrus in the 2 females in which trichomonad pyometra was recognized on clinical examination has been accepted. The animals studied by Hammond or the author were under almost constant daily observation from 8:00 a.m. to 4:30 p.m. The occurrence of estrus was recognized by the usual objective complex: excitement, lowing, discharge of clear mucus from the vulva, "riding" other females, willingness to "stand" while other females mounted and performed mock coital movements, and willingness to accept a male. In a few cases in which estrus was expected but not actually observed, as for example, estrus occurring at night or the so-called "silent heats" or "quiet heats," the collection of a bloody vaginal sample, indicative of a postestrual hemorrhage, was considered as evidence that estrus had occurred two or three days previously. Estrus thus calculated is noted as such.

Exposures were by coitus with bulls known to be infected with *T. foetus* or, in a few instances, by the intravaginal inoculation of cultures, vaginal flushings, or preputial flushings known to contain *T. foetus*. The status of the bulls was determined by the consistent presence of trichomonads in weekly preputial specimens. Bulls 208, 209, and 274 had become infected in outside herds and had been brought to Beltsville. Bull 209 subsequently recovered from infection, spontaneously. The other 3 bulls, 150R, 193, and 207 were experimentally infected, 150R probably through coitus with infected females, and 193 and 207 through the experimental procedures previously described.[9] Each of the bulls was known to be fertile as normal pregnancies were initiated in resistant females by their services during the course of these observations.

Present in the herd at all times were 2 or more susceptible control females either left "open" or served by uninfected bulls. These susceptible controls served as checks on possible nonvenereal transmission of the infection. The resistant females in the herd served as checks, with the above, on the suitability for reproduction of the conditions—frequency of sampling, management, nutrition, etc.—to which these experimental animals were subjected.

RESULTS OF EXPERIMENTS AND DISCUSSION

The history of each female studied is charted in figure 1. The reproductive performance of several cows over a number of years as related to the occurrence, course, effect, and duration of infections is shown. Influence of previously acquired immunity was evident in some females. The times of first appearance of trichomonads in the vaginal samples—termination of prepatent periods and beginning of patent periods—are not shown in the figure.

1—TRANSMISSION OF, AND SUSCEPTIBILITY TO, TRICHOMONIASIS IN:

A) Previously Uninfected Females

Results.— Each of the 29 previously uninfected heifers and cows proved susceptible to infection by *T. foetus* when exposed one or more times.

Twenty-four females were infected by coitus with infected bulls. Twenty became infected following initial exposure; these included 16 nulliparous heifers, 14 of which were virgins, and 4 parous cows. The 4 remaining females became infected following their second exposure; these included 4 nulliparous heifers, 1 of which was a virgin at the time of first exposure. For the purposes of another experiment, 1 of these heifers received, intravaginally, 100 cc. of 40 per cent serum from an "immune" cow prior to both the first and second exposures; the other 3 heifers received no such treatment.

Four heifers were infected by intravaginal inoculation with cultures of *T. foetus;* only 1 failed to become infected following the first inoculation or a series of inoculations. One heifer was infected by coitus with an infected bull and/or inoculation, intravaginally, with cultures of *T. foetus.* Rees and Garlick[10] reported in detail observations on these 5 females.

Summarized, the above shows that a total of only 34 exposures were required to establish infection in all of the group of 29 previously uninfected females. Twenty-four females infected by coitus required a total of 28 exposures; 4 infected by inoculation and 1 by inoculation and/or coitus required a total of six exposures.

Discussion.—These findings clearly indicate the high degree of susceptibility to

trichomoniasis that exists in previously uninfected heifers and cows, and the high level of potential transmission of the infection that occurs at coitus with some infected bulls. Little is known of factors other than immunity that can preclude the establishment of infection in females. It is probable that there are factors peculiar to individual, infected bulls that govern the chance of effective transfer of *T. foetus* from males to females at coitus. The failure of infection to develop in 4 of the females until second exposure may, perhaps, rest primarily with the bulls. Concerning the bulls used in these experiments when coitus with previously uninfected females occurred, the following resulted: 1 bull served 12 females a total of 14 times and produced 12 infections; 1 served 8 a total of nine times and produced eight infections; 1 served 2 a total of two times and produced one infection; 1 served 1 once and produced infection. A bull studied in a commercial dairy herd, whose record is not considered in this paper, caused infections in susceptible females at coitus with much less frequency than those observed in these experiments.

B) Previously Infected Females—Evidence of Acquired Resistance in Such Females

Results.—Twenty previously infected heifers and cows were reëxposed to infection a total of 114 times when served for their successive pregnancies by infected bulls. Four of these females were also exposed once each by intravaginal inoculations with vaginal or preputial flushings containing *T. foetus*, or with cultures. Despite reëxposures, 9 females never became reinfected, although 42 exposures by coitus occurred during intervals varying in individual females from one to five (average, 2.5) years. As a result of 72 reëxposures by coitus, 21 reinfections developed in 11 females; no infections resulted from inoculations. These exposures occurred during intervals varying in individual females from one to seven (average 4.4) years. Three females became reinfected once, 7 became reinfected twice, and 1 became reinfected four times. The intervals between initial and second infections ranged from one to twenty-seven (average, 12.3) months. The intervals between second and third infections ranged from one and a half to fifty-nine (average, 35.7) months.

Discussion.—In direct contrast to the results in which all of 29 previously uninfected females became infected from 34 exposures, are these results in which 11 out of 20 previously infected females developed only 21 infections from 114 exposures. This difference is accepted as being a specific manifestation of acquired immunity.

Insufficient data are available to draw any positive conclusions regarding the duration and intensity of acquired immunity induced in females by *T. foetus* infection. Prolonged initial infection was sometimes followed by an extended period of resistance to reinfection, as shown by 155R, 156R, and 178R which failed to become infected throughout a three- to five-year period of reëxposure. In contrast, 181, 213, and 223, which experienced somewhat shorter initial infections, evidently developed relatively little resistance as they became reinfected from exposure that occurred shortly after the termination of their initial infections. Females 157R, 158R, 160R, and 163R fall between these extremes. Their second infections occurred preceding their second gestations, but were short and of little consequence.

2—PREPATENT PERIODS, PATENT PERIODS, AND PERIODS OF INFECTION IN:

A) Previously Uninfected Females

Results.—A total of 29 females was observed during all or part of their initial infection. The prepatent periods in 16 initial infections resulting from coitus varied from five to twelve (average, 8) days. In the other 13, from which samples were not collected with sufficient frequency to determine the exact date of first appearance of the organisms, *T. foetus* was consistently present before the end of the second week. Patent periods were characterized by a rhythmic appearance and disappearance of *T. foetus* in the vagina. Data relative to these observations and a hypothesis endeavoring to explain the phenomenon were presented in a previous paper.[11]

The periods of infection of 25 initial infections—20 initiated by coitus, four by intravaginal inoculation with culture, and one by coitus and/or inoculation with culture—varied from twenty-eight to 790 (average, 110) days. Disregarding the 3 cases of frank pyometra, the longest period of infection was 150 days and the average for the series was seventy-six days.

Discussion.—Prepatent periods were

characterized by their uniform brevity. Prompt vaginal infection developed in each female. Patent periods and periods of infection varied widely in duration. The 12 cases whose infections were known to have been characterized by the early return of estrus and continuation of the estrual cycle, all had markedly shorter periods of infection than 2 of the 4 cases in which pyometra occurred. The estrual cycles of the latter were decidedly interrupted and their periods of infection were extremely long—216 and 790 days, respectively. The periods of infection of the other 2 females in which pyometra occurred were relatively short. In 1 case, return to estrus and recovery occurred spontaneously on the seventy-seventh day postcoitus. In the other case, trichomonads were not found subsequent to the twenty-eighth day postcoitus. Slaughter on the thirty-eighth day revealed a decomposing conceptus and purulent metritis.

Evidently, the operation of the sexual cycle may exert a profound influence upon the duration of the period of infection. If the estrual cycle is essentially uninterrupted, after an appropriate interval, when sufficient resistance has developed, self-cure results. If the sexual cycle is static, trichomonads may persist in the uterus indefinitely after the death of the conceptus, frank pyometra developing. Evidently, resistance develops but is unable to overcome the mass of organisms. In such cases, restoration of the estrual cycle by therapeutic means is followed by involution and evacuation of the uterus and prompt disappearance of trichomonads. Whether or not pyometra develops, *T. foetus* remaining, is dependent upon the fate of the corpus luteum of pregnancy. The underlying factors involved which govern the fate of the corpus luteum, either allowing it to persist subsequent to death of the conceptus or encouraging its regression, are unknown. Presumably, the stage of gestation when the pregnancy is terminated is one factor. The chance for persistence increases directly as the development of the conceptus advances.

B) Previously Infected Females—Evidence of Acquired Resistance in Such Females

Results.—Eleven females were observed during all or part of 21 reinfections that resulted from coitus. The prepatent periods in 19 of these infections varied from eight to thirty-eight (average, 15) days. The duration of the other two are unknown. The periods of infection in 20 reinfections varied from six to 100 (average, 37) days. In 1 additional female, trichomonad pyometra existed on the day of slaughter, ninety-three days postcoitus.

Discussion.—Prepatent periods in reinfections varied widely in duration. In some females, a short prepatent period was followed by vaginal infection, similar to those observed in initial infections. In others, after a much longer prepatent period, trichomonads appeared suddenly in the vagina in large numbers. These organisms obviously originated from the uterus, thus indicating development of uterine infection without prior or concomitant infection of the vagina. This is believed to be a manifestation of the previously acquired immunity of the vagina, the situation being the same as that in the latter phases of initial infection—when trichomonads discharged from the uterus remained in the vagina for only short periods.[11]

Patent periods of reinfections also varied widely. Generally, however, with a few outstanding exceptions in which pyometra developed, they were shorter than those of initial infections.

It appears that acquired resistance, by depressing the activity of trichomonads, can produce a variety of consequences that influence directly the duration of the period of the parasitic infection. Inhibition of the parasite may allow the development of only an insignificant transient infection which apparently leaves the conceptus unaffected. With contrasting results, inhibition of the parasite may result in very slow continuous development of infection. Death of the conceptus occurred at a relatively advanced stage. Then, abortion or liquefaction of the conceptus may occur and the estrual cycle be resumed, the infection promptly terminating; or the corpus luteum may persist and the estrual cycle remain static, thus allowing development of tricho-

monad pyometra and an indefinite period of parasitic infection. In either of the latter conditions, the period of infection would probably have been shorter had conception not occurred or had the embryo died very early, the estrual cycle being essentially unaffected.

3—EFFECT UPON THE REPRODUCTIVE FUNCTIONS OF THE BREEDING FEMALE, OF:

A) Initial Infection

Results.—Normal pregnancies did not develop in any of 24 females as the result of the coitus responsible for their initial infections. Normal pregnancies did not develop in any of 4 females receiving intravaginal inoculations of cultures about the time of coitus —in three instances with an uninfected bull and in one instance with an infected bull. One female reported by Rees and Garlick[10] experienced infection from inoculation with culture when twenty-one days pregnant but continued and completed her pregnancy.

Sixteen females were observed for occurrence of estrus during infection. Figure 2 shows the durations of interestrual intervals, preceding, during, and following infection of the 12 females in which no extended interruption of the estrual cycle occurred. The remaining 4 females showed marked interruption of their estrual cycles. Of these, 3 experienced frank trichomonad pyometra, 1 returning to estrus on the seventy-seventh day postcoitus, 1 returning to estrus on the 252nd day postcoitus, and the other never returning to estrus during a two-year period of observation. The fourth female had not returned to estrus up to the thirty-seventh day postcoitus, on which day she was slaughtered. The postmortem examination revealed a decomposing conceptus but no *T. foetus*. Some general observations can be made regarding the periodicity of estrus of the 16 females:

1) Preceding infection the interestrual intervals were reasonably uniform (44 of 45 intervals within the "normal" range of eighteen to twenty-four days).

2) During infection the interestrual intervals reflected the influence of the infection. Following the coitus responsible for infection, in 3 of 16 females that developed trichomonad pyometra, and in 1 female that developed septic metritis following *T. foetus* infection, the interestrual intervals were markedly extended; in the remaining females there was moderate or no delay in the return of estrus, indicating either failure to conceive or very early termination of pregnancy. Subsequent to the first return of estrus, both extended and shortened intervals occurred (six of 28 intervals below and five of 28 intervals above the "normal" range of eighteen to twenty-four days).

3) Following infection, the interestrual intervals were more regular than during infection but not as regular as preceding infection (six of 24 intervals were not within the "normal" range of eighteen to twenty-four days).

Females which came in heat during infection did not usually become recognizably pregnant from coitus at this estrus. During initial infection, 10 were served a total of 14 times, one coitus being with an infected bull and the remaining 13 with uninfected bulls; in only one instance was a normal pregnancy apparently initiated. Rees and Garlick (notes in files of the Zoölogical Division) recorded that no *T. foetus* had been found on biweekly examinations of female 156R for a period of one month, subsequent to a period of more than three months of intermittent positive examinations. Coitus then occurred with an uninfected bull. Thereafter, biweekly examinations were negative through the next three weeks. On the twenty-first day postcoitus, Rees and Garlick recorded finding "very few degenerate *T. foetus* in mucous sample." Conception occurred from the above service since, after a proper interval, a calf was born.

The data concerning the periods between the termination of infection and initiation of a normal pregnancy did not give a completely accurate picture of the duration of the period of "postinfection infertility," owing to the fact that the animals were not served at every estrus. Of 13 females in which no complicating reinfection was detected, all of which eventually became pregnant, the period varied from eighteen to 240 (average, 77) days. The total number of services required before all became pregnant was 30; no female was served more than four times.

Discussion.—It is particularly significant that in these experiments none of the females completed a normal pregnancy as a result of the coitus that occurred at the time of exposure to *T. foetus*; also, with one possible exception, coitus occurring during the period of infection did not result in initiation of pregnancy. These findings clearly indicated the highly deleterious effect of typical initial infections upon the early phases of reproduction of the bovine female. Histologic examination of sections taken from uteri of infected females revealed moderate endometritis. Evidently, when infection of the endometrium and placenta was sufficiently intense, any existing pregnancy was terminated. Subsequently, the continuing uterine infection

either interfered with fertilization or prevented nidation.

Frequently, irregularity in the occurrence of estrus has been described as a symptom of diagnostic significance in trichomoniasis of females. Records in these experiments reveal estrual arrhythmia in some instances, but in general these irregularities were not sufficiently pronounced to be of practical significance in diagnosis. Delayed return to estrus, however, did sometimes follow the coitus from which infection resulted. Evidently, that was a manifestation of a pregnancy initiated, but later terminated. No females experienced a shorter than normal interestrual interval subsequent to the coitus responsible for their infections. Sometimes, both extended and shortened interestrual intervals occurred subsequent to the first return of estrus. Figure 2 reveals no significant difference in the duration of those subsequent interestrual intervals, preceded by coitus, as compared with those not so preceded. Therefore, the presence of trichomonad metritis was the only apparent cause for arrythmia of the estrual cycle. In exactly what manner or under what conditions the stability of the endocrine system and periodicity of the estrual cycle might be influenced by *T. foetus* infection is not known.

B) Reinfection—Evidence of Acquired Resistance in Such Females

Results.—Twenty-one reinfections involving 11 females were observed. Five reinfections did not prevent the development of healthy calves initiated by the coitus that produced the reinfection. Two reinfections resulted in abortion of embryos at thirty-four and thirty-five days, respectively, postcoitus. Two reinfections developed into clinically recognizable pyometra. One female returned to estrus on the sixty-seventh day postcoitus and the other still had trichomonad pyometra when slaughtered on the ninety-third day postcoitus. One reinfection evidently resulted in an unobserved abortion, or trichomonad pyometra, which terminated spontaneously, as return to estrus occurred three and a half months postcoitus. Nine reinfections persisted through the first return to estrus three to five weeks postcoitus, thus resembling the majority of initial infections. In one reinfection, the return of estrus occurred at the usual time, but trichomonads were last found on the sixth day postcoitus. One female was slaughtered too soon after exposure to allow drawing any conclusions as to possible influence of reinfection upon reproduction.

The data available are too few to permit analysis for possible different effects of reinfections upon either the regularity of estrual cycles or the intervals between termination of the period of parasitic infection and the time of initiation of a normal pregnancy.

Discussion.—Reinfections frequently differed from initial infections in their effects upon the reproductive functions of affected females. Of 21 reinfections studied, it was highly significant that in 10 cases embryos evidently existed despite concomitant trichomoniasis; 5 healthy calves, 2 abortions, 2 cases of pyometra, and 1 abortion or pyometra resulted. These observations are believed to be evidence of diminished impetus of the infection upon the early phases of reproduction in the bovine female as the result of partial resistance previously acquired. In the 9 cases in which infection persisted through an early first return to estrus, three to five weeks postcoitus, the effect upon the early stages of reproduction cannot be accurately assessed. Probably, some females failed to conceive from causes not related to trichomoniasis. However, the potential, total deleterious effect in the above nine reinfections, and the one instance in which *T. foetus* were not found subsequent to the sixth day postcoitus, was probably minimized owing to the short periods of parasitic infection.

GENERAL DISCUSSION

By discussing the results and conclusions of the foregoing experiments, citing a few incidental field experiences, and reviewing certain literature, several questions—practical by nature or implication—will be answered. These are: (1) Do infected females ever become free of *T. foetus*, and, after calving normally following service by infected bulls, can uninfected bulls safely serve these females? (2) What is the importance of nonvenereal transmission of trichomoniasis? (3) What is the usual clinical evidence in trichomoniasis?

Course of Infection.—Some workers have suggested that females never become free of trichomonad infection but that, rather,

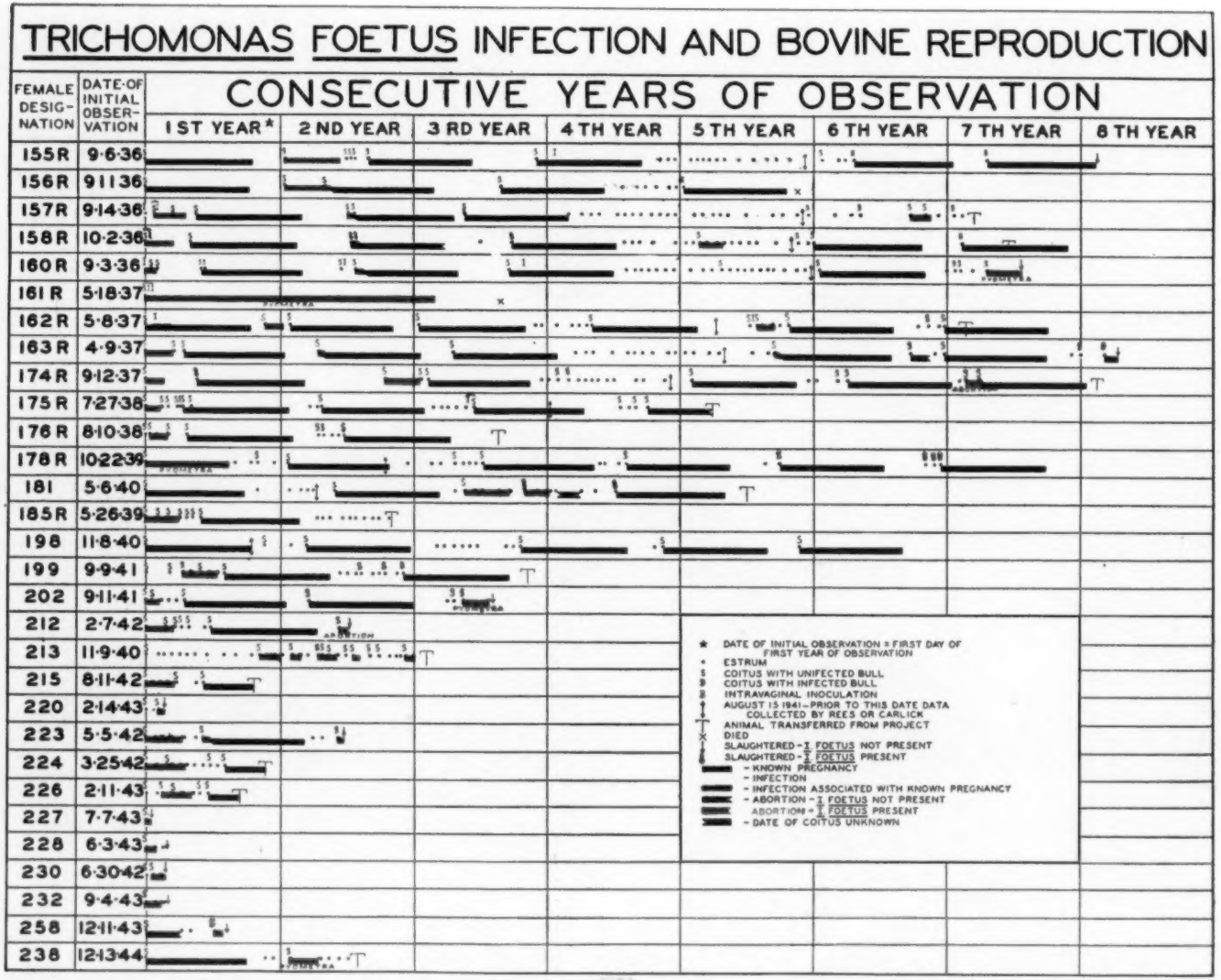

Fig. 1—Records of reproduction of 29 breeding females in an experimental herd are shown. The occurrence of bovine venereal trichomoniasis in these females following exposure to this disease and the delays in producing calves, correlated with infection, are shown. Postinfection immunity is evident in the cases of some

a low level of infection, as in the cases of some sporozoan infections, persists, the resistance evinced being in the nature of premunition. However, no experimental evidence has ever been presented to support this allegation.

The data presented in figure 1 emphasize the distinct nature of each infection in every female studied in these experiments. *Trichomonas foetus* did not appear erratically.

No fact related to the infection in females has more vital and practical significance than whether females may be considered free from *T. foetus* and safe for coitus with uninfected bulls, subsequent to completion of a full term gestation and an appropriate postpartum rest interval (ninety days).

Of the females studied at Beltsville, none were found to harbor trichomonads postpartum. Exceptional cases, however, have been reported. According to a survey of the literature by Morgan,[12] Beller and Schaaf found trichomonads in 1 cow up to three weeks after calving. Küst reported that an infected cow gave birth to a normal calf and, subsequently, a bull became infected by serving this cow. Sachweh reported finding trichomonads more than four weeks postpartum. Salzer reported finding trichomonads subsequent to the second postpartum heat. Kerr and Robertson, Benesch and Wurst, and Rowland presented circumstantial evidence of the presence of trichomonads postpartum. Morgan[13] recorded finding *T. foetus* at parturition and three weeks postpartum in 1 animal and six weeks postpartum in a second one. Gould[14] found trichomonads four weeks postpartum in 1 case and five weeks postpartum in 2 other cases.

The preceding observations evidence the potential infectivity of nonpregnant animals whose latest gestation was preceded or accompanied by infection with *T. foetus*. In the females cited, however, organisms were not found later than six weeks postpartum or the second postpartum estrus.

Only Morgan,[12] and Beach quoted by Morgan,[12] recorded apparent persistence of infection subsequent to the time stated above. Morgan reported that "one cow produced 2 normal calves after service by an infected bull and 1 normal calf after artificial insemination with clean semen. Following the next service with a clean bull, the animal aborted a 4 month fetus." On inquiry Morgan[15] stated: "In checking over the records, I find the 4 months aborted fetus . . . was negative for *Trichomonas foetus*. . . . I will . . . call attention to this erroneous implication." Morgan quoted Beach as stating that "infected cows may carry the organisms for an undetermined length of time without manifesting clinical evidence. Several cows under observation have reproduced normally for two and a half years after having been exposed to an infected bull and then "aborted as a result of trichomoniasis."

On further inquiry, Morgan replied that Beach's records showed the following history of cow 29:

> "Bred 11/21/41 by infected bull (Captain). Trichomonads isolated several times. Calved normally 9/3/42. No organisms found. Bred artificially 12/15/42 (fresh semen in uterus). Clean bull (Ollie). Calved normally 9/2/43. Bred artificially 12/17/43 (fresh semen in uterus). Clean bull (Ollie). Aborted 3/27/44. Positive for *T. foetus*."

Beach's report stands as the only evidence of trichomoniasis persisting through subsequent normal pregnancies that were initiated by uninfected semen. Until this report is confirmed, it seems preferable to proceed on the practical premise that these females are not potential sources of *T. foetus* infection for uninfected bulls. But we should keep in mind the report by Beach, even though the present weight of evidence refutes it.

In summary, these facts are recognized: (1) Persistence of infection postpartum is exceedingly rare. (2) Only one unconfirmed report exists of *T. foetus* being found subsequent to the sixth week or second postpartum estrus. (3) Experience in field herds has not revealed females permanently infective for uninfected bulls.

The following conclusion is drawn: Cows may for practical purposes be considered

free from infection and served by uninfected bulls on the third or subsequent postpartum estrus if they have (*a*) produced a normal full-term calf, (*b*) rested not less than ninety days postpartum, and (*c*) passed at least two periods of estrus since parturition.

Nonvenereal Transmission.—Occasionally, reports of finding *T. foetus* in the genitalia of virgins have appeared in the literature.[12] The practical importance of such observations is obvious. If nonvenereal transmission of this disease is frequent, then isolation of cattle harboring the infection would be essential to control this disease. In the Zoölogical Division's herd, both susceptible and infected animals were at all times in intimate contact, yet only once did an aberrant infection occur. This infection appeared in an 18-month-old virgin heifer during December, 1942. It seems probable that this infection was transmitted mechanically through some fault in technique (sampling or grooming) rather than through contact or insect vectors.

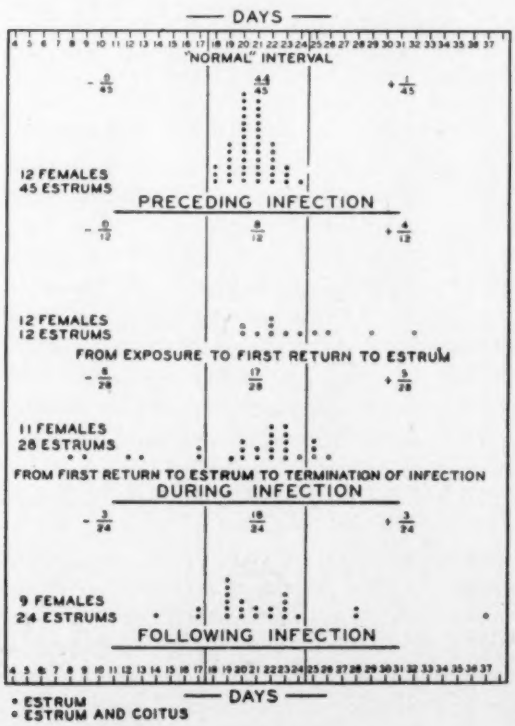

Fig. 2—Comparison of durations of interestrual intervals preceeding, during, and following infection.

On the basis of the observations in the experimental herd and additional field experience, to date, it appears that the probability of nonvenereal transmission under natural conditions is remote and its occurrence exceedingly rare. It was recognized, however, that the disease was readily transmitted by any means of accomplishing mechanical transfer of *T. foetus*. Isolation of potentially infected animals may be theoretically desirable, but in the control of this disease it was not essential. Strict and scrupulous cleanliness in grooming and in carrying out genital examinations is of extreme importance.

Symptomatology.—At no time did any female exhibit evidence of physical discomfort due to trichomoniasis. Records of daily rectal temperatures of 2 females revealed no general febrile reaction. The immediate postcoital (first three or four days) vaginal mucus was no different from that found in sampling unexposed controls subsequent to coitus with uninfected bulls. However, after infection had become established, the presence of a moderate vulvovaginitis and cervicitis was observed at many of the clinical examinations. Slight edema of the labia and perivulvar tissues was noted in the early phases of most infections. Discharges from the genitals were absent or scanty during the course of most infections and would have passed unnoticed under field conditions. Discharge of pus occurred infrequently in cases of trichomonad pyometra. Additional data concerning the pathology of this infection will be presented in a later paper.

In diagnosis, existence of any of the preceding symptoms is of little significance since none are consistent, specific, or even particularly characteristic.

Of the 50 distinct infections studied in this series, 2 were associated with abortion of recognizable embryos and 5 with development of frank trichomonad pyometra, diagnosed on clinical examination. The history of 1 other female indicated that either an unobserved abortion occurred or a pyometra was corrected spontaneously. Of the remaining 42 infections, six obviously

failed to interfere with pregnancy; 35 were characterized by continuation of the estrual cycle and a period of "temporary infertility"; and 1 was terminated by slaughter of the female too early for any conclusions to be drawn.

It is unfortunate that bovine venereal trichomoniasis has been so generally accepted as a disease characterized chiefly by symptoms of abortion and pyometra. If this were so, cursory examination would suffice in making a herd diagnosis of infection. Actually, the data presented in this paper, and the results of field studies in five extensively infected herds in Maryland (unpublished data in files of Zoölogical Division) involving almost 300 additional beef and dairy cattle, clearly refute this conception. For example, one herd manager was aware of only 1 early abortion having occurred and he had witnessed only 1 cow, subsequently found on clinical examination to have trichomonad pyometra, discharging pus. The other herd managers and assistants—all average or above in diligence—reported observing no abortions or pus discharges. To them, the primary reproductive fault was failure of most females to become normally pregnant, despite repeated services, or failure to become pregnant until served many times. Usually, some females were reported to have returned to estrus after they had been thought to be pregnant.

When trichomonad abortions do occur, they are most frequently embryonic abortions, that is, abortions at fifteen to eighty days of gestation rather than fetal abortions at eighty or more days. The former are likely to pass unnoticed in ordinary herds.

Trichomonas foetus characteristically terminates pregnancies so early in gestation that a recognizable conceptus is not aborted. Evidently, intrauterine maceration is frequent.

SUMMARY AND CONCLUSIONS

1) Heifers and cows previously uninfected with *Trichomonas foetus* are highly susceptible to this pathogenic protozoan. Infection usually confers a relative degree to temporary resistance to reinfection.

2) Trichomoniasis in females is essentially self-limiting. The occurrence of *T. foetus* in the vagina and the persistence of infection are influenced by the sexual cycle and by acquired immunity.

3) Early termination of pregnancy with early return to estrus and continuing catarrhal metritis is the most common consequence of trichomoniasis in the female. Abortion of the conceptus during its embryonal or early fetal state with return to estrus, production of frank trichomonad pyometra and static condition of the estrual cycle, and successful gestation despite concomitant infection also may occur.

4) The results of *T. foetus* infections upon pregnancies vary in accordance with the activity of the infections. Active infections ordinarily terminate pregnancies early. Less active infections, usually limited by previously acquired immunity, may not prevent the conceptus from developing to an advanced stage. Thus, abortions of recognizable embryos and fetuses, pyometras, and successful gestations despite infections are more likely to occur in cows experiencing reinfections—in herds where trichomoniasis has been present for several years.

5) It is concluded that infected females may be considered free of *T. foetus* and safely allowed coitus with uninfected bulls if they have completed (*a*) a normal pregnancy, (*b*) a ninety-day postpartum rest period, and (*c*) three periods of estrus.

6) Nonvenereal transmission of trichomoniasis under natural conditions is very rare. Isolation of infected animals is not essential in controlling the disease.

7) Such objective symptoms as genital discharges and abortions are of limited importance in practical diagnosis of trichomoniasis not only because they usually pass unnoticed, but because such occurrences are relatively infrequent.

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Hexachloroethane-Bentonite Suspension for Controlling the Common Liver Fluke, Fasciola Hepatica, in Cattle in the Gulf Coast Region of Texas

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Angleton, Texas

HEXACHLOROETHANE in suspension with bentonite and water was reported by Olsen[30] to be efficacious and relatively safe, when administered as a drench, for removing the common liver fluke of cattle.

Since the appearance of the preliminary announcement of the fasciolicidal efficacy of the suspension, additional data have been obtained on this method of controlling flukes in cattle. The purpose of this paper is to report the information accumulated by experiments designed to evaluate the method in cattle under ranch conditions in the coastal region of Texas.

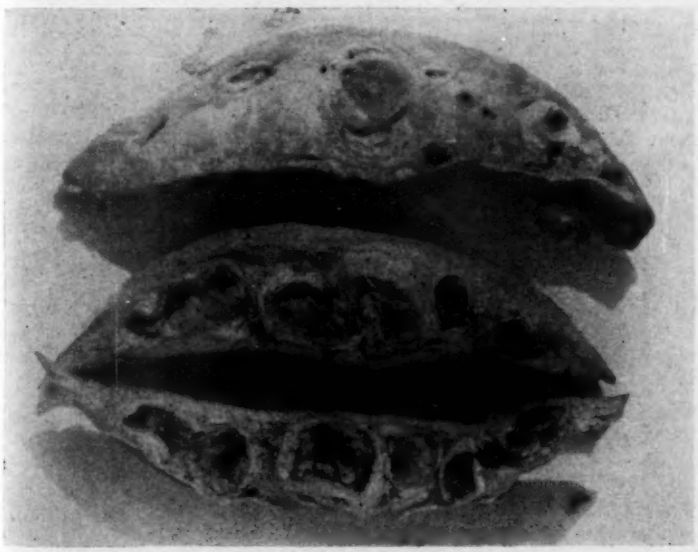


Fig. 1—Sections from ventral lobe of the liver of a cow showing macroscopic pathology resulting from liver fluke infection. Extensive connective tissue hyperplasia has replaced all liver parenchyma. The greatly enlarged and thickened bile ducts were filled with both organic and inorganic material, together with flukes.

REVIEW OF LITERATURE

Hexachloroethane was first tested as an anthelmintic by Hall and Cram,[13] who administered it to dogs in crystalline form in capsules, and in solution in castor oil, oil of chenopodium, and carbon tetrachloride. They concluded, from their experiments, that hexachloroethane did not remove hookworms from dogs, because of its low solubility in water.

According to Nöller and Schmid[26] and Nöller,[25] hexachloroethane was first investigated as a fasciolicide for Merck and Company by Sprehn. Opel[27] stated that these investigations were never concluded, and its value in that rôle was not demonstrated.

Hexachloroethane was introduced as a fasciolicide in cattle by Thienel in Germany[41] under the trade name Neoserapis. According to Nöller, Gluscke, and Schmid, [25] Neoserapis contained principally hexachloroethane dissolved in tetrachlorethylene. It was administered either as a drench or in gelatin capsules. Medication with Neoserapis was completed in three successive days.

Of the many proprietary fasciolicides containing hexachloroethane introduced in Germany subsequent to the appearance of Neoserapis,[4, 18, 19] only two seemed to have gained general use in cattle therapy. One of these preparations, Distol comb., prepared by Marek,[23] was a mixture of 92 per cent powdered hexachloroethane and 8 per cent crude

From the Zoölogical Division, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture.

Acknowledgment and appreciation are expressed to the ranchers whose coöperation made this study possible.

filicin;[39] it was dispensed in capsules. The other preparation, Igitol, prepared by the Behringwerke at Marburg,[39] consisted of powdered hexachloroethane and starch pressed into boluses.[38] According to Rosenberger and Slesic,[39] who made comparative tests on cattle with Distol comb. and Igitol, the latter was preferable because it could be given in a single dose, whereas Distol comb. was administered over a period of three to four consecutive days.

In Germany, Nöller, Flietner, and Schmid[27] showed that cattle could tolerate, without ill effects, up to twice the required dose of chemically pure, crystalline hexachloroethane given in gelatin capsules. In one instance, the double dose was repeated in twelve days without untoward results. These investigators stated that oily foods should be withheld for several days prior to, and during the course of, treatment.

In the Lombardy section of Italy, where fascioliasis is a serious disease of cattle, Vianello[47] made boluses weighing 40 Gm. each from hexachloroethane (60 per cent by weight) and vegetable oils and soap which disintegrated in water at 37 C. Doses of 70 Gm. of hexachloroethane for each 220 lb. of body weight were well tolerated by cattle, but 25 Gm., the amount contained in one bolus, for each 220 lb. of body weight was therapeutically sufficient. It was recommended that medication be repeated bimonthly.

In Hawaii, Alicata[1] prepared the medicament by placing 10 Gm. of finely ground hexachloroethane and 1.75 Gm. of kamala extract in hard gelatin capsules. Medication at the rate of one capsule for each 70 lb. of body weight was given on two successive days.

In southern Texas, Olsen[35] prepared an aqueous suspension by mixing 500 Gm. of finely ground hexachloroethane, 50 Gm. of bentonite, ¼ to ½ teaspoonful of white flour, and water sufficient (750 cc.) to make 1,000 cc. In the beginning of this investigation, the suspension was administered at the rate of 20 cc. per 100 lb. of body weight but later a dose of 200 cc. for grown animals and 100 cc. for yearlings was recommended and used.[32] Treatment was completed with a single dose of the suspension without fasting the cattle.

MATERIAL AND METHODS

The cattle used in this study were obtained from ranches in the coastal prairie of Texas. They included animals of both the European and Asiatic breeds.

Results of medication were determined by (1) pre- and postmedication egg counts on fecal specimens taken from the rectum of cattle medicated once; (2) periodic egg counts on cattle kept on the same pasture and medicated twice annually for three consecutive years; (3) postmortem examinations of livers of medicated and unmedicated cattle; (4) weight studies of animals prior to treatment and again about seven months later; and (5) effects of the hexachloroethane therapy in cases of clinical fascioliasis.

The technique for making egg counts was similar to the one reported for sheep,[35] except that in the case of cattle 30 Gm. of feces were weighed into the flask. For counting, 5-cc. samples of the fecal suspension, containing 0.5 Gm. of the original fecal material, were used.

Samples larger than 5 cc. of fecal suspension do not clear satisfactorily in 15-cc. tubes. Two samples may be counted at a considerable saving of time by combining them in one counting chamber after they have been cleared in the usual manner.

Concentration of liver-fluke eggs by means of flotation, modified from Brüggemann's[6] method, was made by centrifuging in an electrically driven centrifuge at about 2,000 r.p.m. for two to three minutes, the precipitate from 2.5-cc. samples of fecal suspension in 15-cc. tubes filled with sodium silicate solution having a specific gravity of 1.28 to 1.30. Sodium silicate of this specific gravity levitates most of the eggs but allows the greater portion of the débris to precipitate. Such detritus as arises to the surface is rendered quite clear by the fluid. The eggs were looped from the surface of the fluid to a slide which was marked by transverse lines. Although the liver-fluke eggs were collapsed by the action of the sodium silicate, they could be readily identified and differentiated from those of the rumen fluke.

RESULTS OF TREATMENTS

Egg Counts.—Of 463 mature cattle positive by fecal examination for liver flukes at the time of medication, eggs were found in only 35 animals two to three weeks after one treatment (table 1). The pretreatment egg counts of the 463 animals averaged 26.4 ± 40.4 (standard deviation) per gram of feces and the post-treatment counts of the 35 animals that remained positive averaged 4.9 ± 5.7 (s.d.) per gram.

Because it is more difficult to find eggs in the large fecal mass of cattle by routine examination when they are greatly reduced, as occurs following medication with hexachloroethane, it is probable that small numbers of flukes might survive treatment and remain undetected. The disappearance of eggs in about 92 per cent of the cattle, however, points to the high efficacy of hexa-

chloroethane suspension in destroying egg-laying flukes.

Dilution egg counts indicated that light infections occurred in most of the cattle with only a relatively few harboring heavy infections. A similar egg distribution was found by Scott[40] for hookworm infections in man. Out of 933 fecal specimens positive for flukes, approximately 91 per cent had 50 or fewer eggs per gram of feces (graph 2). Only about 6 per cent of the cattle had sufficiently high egg counts to indicate the probability of clinical fascioliasis.

Effect of Periodic Medication in Controlling Liver Flukes.—Two herds of cattle, one of Brahman (Zebu)-Hereford cross, known as Brafords, numbering about 300 animals, and one of Herefords, numbering about 350, were provided by ranchers for treatment semiannually with hexachloroethane suspension. The purpose of this study was to determine to what extent liver flukes could be controlled in a given locality when all the cattle were subjected to a regimen of periodic medication. Each herd was confined to its own pasture throughout the three years that the study was conducted. All cattle put on the pastures during this time were medicated prior to admission.

Medication of these two herds was done, for the most part, in the spring or early summer, and again late in the fall. The status of the infections was determined by egg counts made from random fecal samples obtained at the time of each medication, and also at intervals between treatments.

The Braford cattle were kept on a river bottom pasture in Brazoria County. They grazed on both open and wooded areas, the former having been cleared of forest many years ago. The average annual rainfall in the area is approximately 47 in.[7] The Hereford cattle grazed on open prairie country in Refugio County, which is about 150 mi. southwest of the Brazoria County pasture. The average annual rainfall in that region is between 31 and 33 in.[7] Rainfall in both locations exceeded the mean annual precipitation by 10 in. or more during the course of this study.

In the Braford cattle, 75 per cent of the 98 grown animals sampled were infected with liver flukes, as determined by fecal examinations, at the beginning of this study. Individual infections ranged from 2 to 168 eggs per gram of feces at the time of the initial treatment. The mean number of eggs per gram of feces per animal was 13.8 ± 22 (s.d.). During the course of medication, the average egg count steadily declined to a mean low of 0.1 ± 0.3 (s.d.) eggs per gram of feces (table 2). While the number of eggs per gram of feces was steady in its decline, the incidence of infection in the herd showed great fluctuation (graph 3). Within the six-month intervals between medication, the incidence of infection generally increased but dropped sharply following each treatment (graph 3). At

TABLE I—Results of the Treatment of 463 Cattle with Hexachloroethane-Bentonite-Water Suspension for the Removal of the Common Liver Fluke, Fasciola Hepatica, According to Egg Counts

Animals Treated (No.)	Eggs/Gm. Feces/Animal Before Treatment* (No.)	Animal		Eggs/Gm. Feces/Animal After Treatment (No.)
		Neg.	Pos.	
		After Treatment (No.)	(No.)	
79	2	73	6	2, 2, 2, 2, 2, 4
73	4	70	3	2, 4, 8
21	6	21	0	0
83	8-12	76	7	4, 4, 4, 4, 4, 6, 8
36	14-18	34	2	2, 4
51	20-30	46	5	2, 4, 4, 4, 4
49	32-50	46	3	4, 4, 36
30	52-78	27	3	2, 4, 4
25	80-124	21	4	2, 4, 8, 8
10	120-198	10	0	0
6	200-318	34	2	4, 4
463		428	35	

*Logarithm of eggs per cubic centimeter when factor is 2, as suggested by Scott.[41]

one time (Nov. 3, 1945), the number of infected cattle was higher than when the study began. The mean egg count, however, was 7.1 ± 7.5 (s.d.), as compared with the original count of 13.8 ± 22 (s.d.).

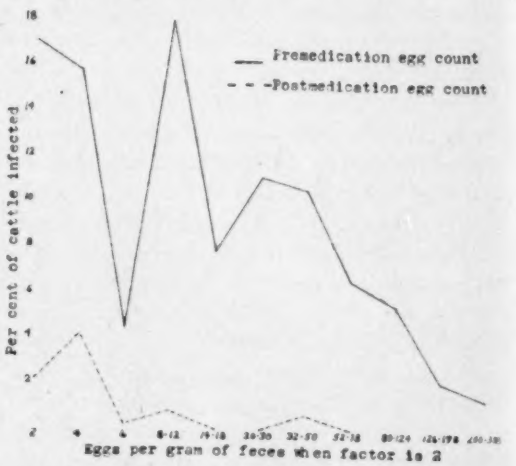
The trend of the fluke population in the Hereford cattle was similar to that in the Brafords in that the incidence of infection increased during the interval between each treatment. On five occasions, the incidence of infection exceeded the original one (table 3, graph 4). At one time (Nov. 14, 1944), 87.5 per cent were infected with a mean of 21 ± 25.3 (s.d.) eggs per gram of feces as compared with the original infection of 35.3 per cent and a mean egg count of 2.9 ± 3.7 (s.d.).

These data showed that semi-annual medication with hexachloroethane of all cattle confined to a particular pasture did not prevent recurrence of a high incidence of liver-fluke infection. However, medication did reduce the number of flukes in the Braford cattle and precluded clinical fascioliasis in both herds, where it had been known to occur prior to the beginning of treatment.

Three factors appear responsible for recurrence of the high incidence of infection in these two herds of cattle. They are (1) a residue of flukes remaining in the cattle after medication, (2) presence of the snail intermediate host on the pastures, and (3) the presence of a reservoir host, other than cattle, from which infestation of the pastures was maintained.

Postmedication egg counts, as well as postmortem examinations to be discussed elsewhere in this paper, showed that a residue of adult flukes remained in the cattle following treatment (table 1, graph 1). So long as flukes survive and the snail intermediate host is present on the pasture, infections are likely to recur in cattle grazing on it.[39]

During periods when the pastures are dry, the snail intermediate host (*Stagnicola bulimoides techella* (Hald.)) of the fluke in the Gulf Coast region estivates in the soil.[31] Infections in snails survive the summer droughts, as shown by the presence of fully



Graph 1—Distribution of egg counts in 463 cattle positive for liver flukes at time of medication with hexachloroethane suspension and again two to three weeks after medication.

TABLE 2—Liver Fluke Egg Counts in Braford Cattle on River Bottom Pasture, Brazoria County, Texas, During the Three-Year Period of Semiannual Medication with Hexachloroethane Suspension

Date of treatment and sampling	Cattle				Fluke eggs		
	Exam. (No.)	Pos. (No.)	Neg. (No.)	Infected (%)	Total (No.)	Maximum observed/gm. feces (No.)	Mean/gm. feces/cow (No.)
1-28-43*	98	74	24	75.5	1,348	168	13.75 ± 22 (s.d.)
9-30-43*	135	56	79	41.0	?†	?	?
1-26-44	75	13	62	17.3	68	12	5.8 ± 5.4
3-16-44	49	12	37	24.5	56	14	1.1 ± 2.9
7-12-44*	49	30	19	61.0	152	16	3.1 ± 4.5
8-12-44	50	6	44	12.0	12	2	0.24 ± 0.7
11- 6-44*	24	12	12	50.0	50	14	2.1 ± 3.3
1-19-45	20	3	17	15.0	1	1	0.05 ± 0.1
6-22-45*	20	5	15	25.0	28	18	1.4 ± 4.1
11- 3-45	40	32	8	80.0	284	34	7.1 ± 7.5
1- 1-46	11	2	9	18.2	2	1	0.9 ± 0.9
5-10-46*	20	2	18	10.0	2	1	0.1 ± 0.3

* Date medicated.
† Count not made.

developed cercariae, identified as *Fasciola hepatica*, found in snails several days after being activated by standing water for the first time in several months.

Refugio County, where the Hereford cattle grazed, is in the range of the jack rabbit (*Lepus californicus merriami* (Mearns)).[23] Jack rabbits in this area have a high incidence of infection with liver flukes. Of 178 rabbits examined postmortem by the writer during the early part of the summer of 1946, 58 (32.6%) were infected with the common liver fluke. There were many more rabbits than cattle in this area, and their droppings were closely dispersed over the choice grazing places.

Rabbits of the genus Sylvilagus (cotton tails and swamp rabbits) from other sections of the Gulf Coast[8] and the vicinity of the ranch where the Braford cattle grazed have likewise been found to be infected with liver flukes. Of 6 rabbits examined from this general area, 1 was infected.*

Postmortem Examinations.—In order to determine with greater accuracy than through the use of the egg counting method the efficacy of medication with hexachloroethane suspension in removing liver flukes from cattle, postmortem examinations were made on treated and untreated animals from the same pastures.[33] The cattle were selected for slaughter and medicated one to two weeks before delivery to the packing plant. In the interim between medication and slaughter, they remained on their pastures.

The cattle examined postmortem included 143 grown and 29 young animals. The calves were unweaned and averaged about 8 months old. Of the 143 grown cattle, 91 were medicated with 200 cc. of hexachloroethane suspension and 52 remained unmedicated. Fifteen of the calves were treated with 100 cc. of the medicament, and 14 remained untreated.

All animals were slaughtered within twenty-four hours after reaching the packing plant. The livers were examined immediately after slaughter, and the flukes were collected and divided into sexually mature and immature individuals.

The results obtained from examination of the livers of the grown cattle showed an average of 23.9 ± 23.9 (s.d.) flukes of all sizes per untreated animal as compared with 3.3 ± 10.6 (s.d.) per treated animal, and 20.8 ± 28.6 (s.d.) and 1.4 ± 3.6 (s.d.) mature flukes per untreated and treated animal, respectively. The number of young flukes per animal was nearly equal in both groups. These data are summarized in table 4 and graph 5.

Comparison of the percentage of positive animals among the unmedicated and medicated cattle was made to determine whether the difference obtained might be the result of random sampling or of the fasciolicidal effect of the hexachloroethane.†

TABLE 3—Liver Fluke Egg Counts in Hereford Cattle on Prairie Pasture, Refugio County, Texas, During the Three-Year Period of Semiannual Medication with Hexachloroethane Suspension

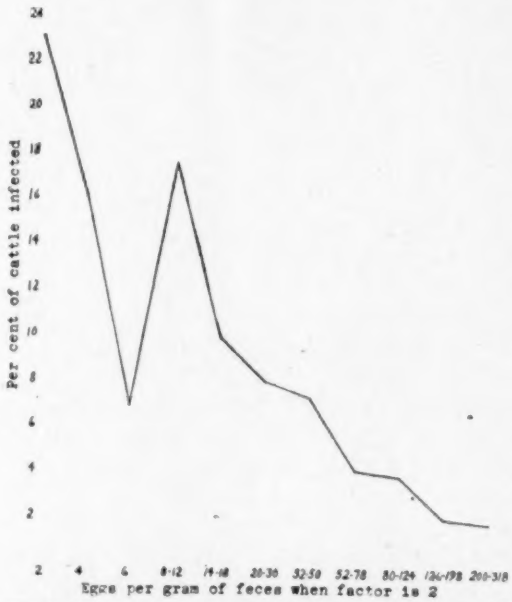
Date of treatment and sampling	Cattle				Fluke eggs		
	Exam. (No.)	Pos. (No.)	Neg. (No.)	Infected (%)	Total (No.)	Maximum observed/gm. feces (No.)	Mean/gm. feces/cow (No.)
8- 9-43*	113	40	73	35.3	166	30	2.9 ± 3.7 (s.d.)
8-24-43	38	1	32	3.0	2	2	0.06 ± 0.35
4-21-44*	50	25	25	50.0	75	27	1.5 ± 3.9
5- 6-44	50	0	50	0	0	0	0
7-19-44	50	28	22	56.0	140	28	2.8 ± 4.8
11-14-44*	24	21	3	87.5	504	106	21.0 ± 25.3
1-24-45	27	10	17	37.0	12	2	2.3 ± 1.9
5- 7-45*	19	4	15	21.0	16	6	0.84 ± 1.8
11-15-45*	45	37	8	82.2	340	42	7.6 ± 9.5
5- 3-46*	40	9	31	22.5	30	6	1.3 ± 1.7
8- 8-46*	20	5	15	25.0	8	2	1.6 ± 1.4

* Date medicated.

*Reported through the courtesy of Dr. R. L. Stanger, Brazoria, Texas, who collected and examined the infected rabbit..

†Appreciation is expressed to Dr. J. Allen Scott, Medical School, University of Texas, Galveston, for assistance with the statistical work.

In the total fluke population, about 85 per cent of the unmedicated and 44 per cent of the medicated animals were positive for flukes. The difference between the two groups is 40.7 ± 7.7 per cent (standard error difference). This difference of five



Graph 2—Distribution of intensity of infection with Fasciola hepatica in 933 Gulf Coast cattle, according to egg counts.

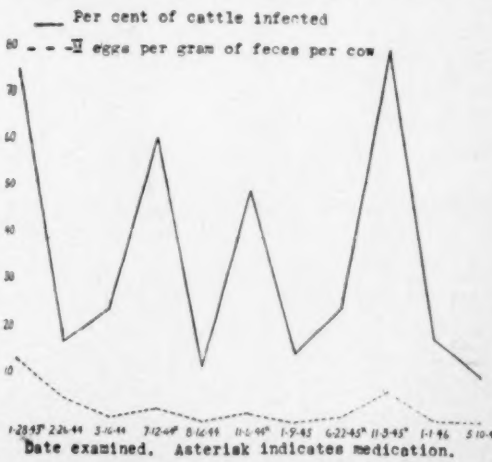
times its standard error indicates a high probability that the smaller number of flukes in favor of the medicated cattle was not due to random sampling.

On the mature flukes present, the results were similar to those obtained for the total number of flukes. In this category, approximately 73 per cent of the untreated and 30 per cent of the treated animals were positive for flukes. The difference between them is 43 ± 7.7 per cent (s.e. difference). Again, the difference in favor of medication does not appear to be due to chance.

In the case of the immature flukes, however, the difference in the percentage of positive animals between the two groups is very small. There were about 37 per cent and 20 per cent positive in the unmedicated and medicated cattle, respectively. The difference between them is 17 ± 7.4 per cent (s.e. difference). Since this difference is only slightly more than twice its standard error, the probability of its being due to chance involved in random sampling makes definite conclusions uncertain.

Adult flukes surviving medication were found more often in livers or sections of livers showing marked pathologic changes due to fluke activity than in less severely damaged livers. Frequent survival of flukes in livers showing marked damage suggested the possibility of some relationship between the pronounced pathologic changes and fasciolicidal efficacy of the drug.[12,27,45]

Hexachloroethane absorbed from the intestine by the blood and eliminated through the bile kills flukes in the choleic ducts when the concentration of the drug is sufficiently high.[20,45] In sections of the liver where extensive pathologic changes pro-



Graph 3—Fluctuation of incidence of infection with Fasciola hepatica and average egg counts in Braford cattle on river bottom pasture, Brazoria County, Texas, during regimen of medication with hexachloroethane suspension semiannually for three years.

voked by fluke activity occur, such as parenchymatous tissue atrophy and connective tissue hyperplasia,[15,24] the excretory functions may be diminished, if not destroyed. Hence, flukes in livers or parts of livers where dysfunction occurs due to advanced pathologic changes are more likely to survive medication than flukes in livers less severely damaged.

In order to compare the efficacy of hexachloroethane in livers whose physiologic

functions were not impaired by severe pathologic changes, such as sclerosis and hypertrophied bile ducts resulting from fascioliasis, 29 unweaned calves were obtained for medication from a ranch where the incidence of liver fluke infection was high.

Among the 15 medicated calves, one to four fluke eggs per gram of feces were found in 9 individuals; no eggs were found in 6 animals. Of the 14 untreated calves, 7 were unavailable for examination, 3 showed no eggs, and 4 showed two to four eggs per gram (table 5). The calves were slaughtered one week after medication. Postmortem examination of the livers of the treated animals showed no living flukes. One calf, which was found to be negative on the basis of egg counts, had one dead and partially decomposed fluke in its liver. Of the 14 untreated calves, 12 harbored from 1 to 20 living adult flukes in their livers (table 5). Gross pathologic changes in the livers, when present, were similar in both groups of calves. They consisted primarily of slight hypertrophy of that portion of the bile ducts occupied by the flukes.

Weight Studies.—Preliminary observations were made, under field conditions, on gains of weight in untreated and treated cattle. The experimental animals included 40 Hereford cows, 3 to 4 years old, and their 8- to 12-week-old steer calves. The cows were divided prior to weighing, on the basis of physical similarity, into two groups of 20 cows and calves each. Due to difficulties in weighing the animals individually, each group was weighed as a unit. The data are treated on the basis of average weights.

The cattle were under observation from March 13 to October 3, a period of 205 days. During this time, they grazed together on the same pasture. (It was on this ranch where jack rabbits were found so generally infected with liver flukes.) On March 13 and again on May 16, 20 cows were medicated with 200 cc. of hexachloroethane suspension; on the latter date, their calves received a single dose of 100 cc. The other 20 cows and calves were not treated.

All the cows were infected with liver flukes at the beginning of the study. The untreated cows averaged 7.7 ± 8.6 (s.d.) fluke eggs per gram of feces and the treated ones averaged 5.1 ± 3.4 (s.d.) eggs.

At the conclusion of the experiment, an average of 6.4 ± 6.2 (s.d.) eggs per gram of feces was found in 15 of 20 untreated cattle, and 2 ± 3.2 (s.d.) eggs in 6 of 13 treated cows. Fecal samples were not available from the remainder of the cows. Three calves from each group were lightly infected with flukes.

The initial and final weights of these two groups of cattle are given in table 6. The untreated cows and calves, which were heavier than the treated ones at the beginning of the experiment, maintained their superior weight throughout the period of observations. Their daily gains were slight-

TABLE 4—Distribution at Postmortem Examination of (1) Total Number of Flukes, (2) Mature Flukes, and (3) Immature Flukes in 52 Unmedicated Grown Cattle and 91 Grown Cattle Medicated with Hexachloroethane Suspension

Cattle	Number of flukes per animal										Total flukes (No.)	Total cattle (No.)	Mean flukes /animal (No.)
	0	1	2	3	4-6	7-9	10-15	16-50	51-79	80-128			
1) *Total Flukes*													
Untreated cattle	8	8	1	2	5	3	5	11	5	4	1,243	52	23.9 ± 23.9*
% of cattle	15.4	15.4	1.9	3.8	9.6	5.8	9.6	21.2	9.6	7.7			
Treated cattle	51	7	7	6	11	2	4	2	0	1	304	91	3.3 ± 10.6
% of cattle	56.0	7.7	7.7	6.6	12.1	2.2	4.4	2.2	0	1.1			
2) *Mature Flukes*													
Untreated cattle	14	8	0	1	4	3	5	10	4	3	1,080	52	20.8 ± 28.6
% of cattle	26.9	15.4	0	1.9	7.7	5.8	9.6	19.2	7.7	5.8			
Treated cattle	64	3	8	2	11	1	1	1	0	0	128	91	1.4 ± 3.6
% of cattle	70.3	3.3	8.8	2.2	12.1	1.1	1.1	1.1	0	0			
3) *Immature Flukes*													
Untreated cattle	34	4	4	4	2	1	1	1	0	1	183	52	3.5 ± 13.6
% of cattle	65.4	7.7	7.7	7.7	3.8	1.9	1.9	1.9	0	1.9			
Treated cattle	73	7	1	3	2	1	2	1	0	1	176	91	1.9 ± 10.2
% of cattle	80.2	7.7	1.1	3.3	2.2	1.1	2.2	1.1	0	1.1			

* Standard deviation.

ly in excess of those for the treated animals.

The results of this limited experiment indicated that under favorable nutritional and weather conditions, such as these cattle experienced, gains in weight did not follow medication with hexachloroethane of cows lightly infected with liver flukes, nor their nursing calves, over gains in similarly infected but unmedicated cows and their calves.

In contrast to the results obtained by the present writer, other writers have reported gains in weight following medication and removal of the flukes.

According to Thienel,[45] questionnaires returned by 26 official and practising veterinarians in Germany stated that Neoserapis was highly efficacious in removing flukes. All workers reporting agreed the nutritional condition of the cattle improved greatly following treatment due to increased appetite and better utilization of feed.

It was the opinion of Shaw[24] at one time that losses in milk and flesh in the total cattle population of Oregon due to liver flukes probably exceeded those resulting from the more severe and frequently fatal form of fascioliasis of sheep in that state. In 5 steers lightly infected with flukes and kept under optimal feedlot conditions for 104 to 112 days, Shaw[42,43] presented weights showing average gains in favor of animals medicated with hexachloroethane suspension. Moreover, these gains occurred even though only 2 of the animals became completely free of flukes. It was Shaw's[42] opinion that extensive liver injury did not necessarily depend on a large number of flukes. Other investigators, however, believed there is a relationship between the number of flukes present and the degree of injury to the liver.[15] According to Frederick,[11] flukes derange normal liver function, thus affecting adversely the general well-being and health of cattle. Infected animals made less favorable gains under feedlot conditions than comparable uninfected ones. Alicata[2] stated that infected cattle improved in appearance and weight following removal of flukes by medication with hexachloroethane. Kirsten[17] and Nöller, Flietner, and Schmid,[27] discussed elsewhere in this paper, reported outstanding gains following medication with hexachloroethane of cattle debilitated as the result of fascioliasis. Ranchers who have medicated their cattle regularly with hexachloroethane suspension, both in the Gulf Coast and Rocky Mountain regions, have reported marked improvement in the appearance and condition of their cattle. Where clinical fascioliasis was common prior to the program of medication, they no longer observe symptoms of the disease.

HEXACHLOROETHANE IN THE TREATMENT OF CLINICAL FASCIOLIASIS

The etiology of fascioliasis involves a complexity of factors that lead to tissue destruction and change, hepatic dysfunction, and cachexia. Using chemical and pharmaceutical products obtained from liver flukes, Flury and Leeb[10] produced symptoms of the disease in experimental

TABLE 5—Liver Flukes Found at Postmortem Examination of 8-Month-Old Calves, 15 of Which Received Hexachloroethane Suspension One Week Prior to Slaughter, and 14 of Which Received No Medicament

GROUP	CALVES EXAMINED (NO.)	EGGS/GM FECES (NO.)	HEXACHLOROETHANE REC'D (GM.)	FLUKES/ANIMAL AT AUTOPSY		TOTAL FLUKES (NO.)	MEAN FLUKES /CALF (NO.)
				IMMATURE (NO.)	MATURE (NO.)		
Treated	6	0	50	0	1*	1*	
	5	1	50	0	0	0	
	2	2	50	0	0	0	
	2	4	50	0	0	0	
Total	15				1	1	0.064
Untreated	7	?†	0	1,1,2,4	1, 20	29	
	3	0	0	0	1, 1	2	
	3	2	0	0	5, 8, 9	22	
	1	4	0	0	3	3	
Total	14			8	48	56	4

* Fluke dead in bile duct with posterior half of body decomposed.
† Fecal samples not available for examination.

animals; this led them to believe that fascioliasis was the combined result of (1) tissue changes following destruction of liver parenchyma, (2) toxic effects of excretory products of the flukes, (3) the probability that continuous disintegration of foreign albumins in the liver produced cachexia, and (4) poisoning of the liver. Removal of flukes from animals suffering from clinical fascioliasis should, therefore, enable them to eliminate the accumulated toxic substances and return to their normal health.

During the course of this study, many animals showing clinical fascioliasis have been available for medication with hexachloroethane. The disease was diagnosed or confirmed on the basis of (1) external symptoms of emaciation, debilitation, and exhaustion, together with occasional serous diarrhea and submandibular edema, (2) abnormally high liver fluke egg counts, 100 to 308 per gram of feces, without accompanying nematode infections of significance, and (3) rapid recovery after medication with hexachloroethane and expulsion of flukes. These animals were losing weight under pasture and feedlot conditions that maintained other cattle in good physical vigor and flesh. In enfeebled animals, breeding in both bulls and cows had ceased. Following medication and removal of the flukes, as evidenced by postmedication egg counts, these animals usually experienced early recovery of appetite, together with greatly improved physical condition and restored fecundity.[32] Occasionally, however, the removal of exceedingly heavy fluke infections from debilitated animals, as indicated by pre- and postmedication egg counts, and finally autopsy, failed to result in recovery.[32]

Other investigators have reported similar results in the medication of cattle suffering from clinical fascioliasis.

In 4 young cattle so weak from fascioliasis they were barely able to walk to the water trough, Kirsten[17] reported rapid recovery of strength following medication with Neoserapis, together with gains of 40, 42, 49, and 62 lb. in three weeks. In another instance, treatment of 4 animals so enfeebled from fluke infection they could rise only with assistance resulted in restored appetites and gains of 25, 31, 32, and 41 lb. in eighteen days. An 8-year old cow that had lost 100 to 150 lb. in six to eight weeks due to fluke infection gained 72 lb. in five weeks following medication. It was Kirsten's opinion that fascioliasis was the effect of accumulated toxins resulting from the flukes, as evidenced by the persistent diarrhea, and that recovery followed removal of the flukes and elimination of the poisonous substances.

In 23 young cattle debilitated as a result of liver fluke disease, Nöller, Flietner, and Schmid[27] reported rapid improvement following medication with crystalline hexachloroethane in gelatin capsules, with an average gain of 59 lb. in forty days; 1 animal gained 88 lb.

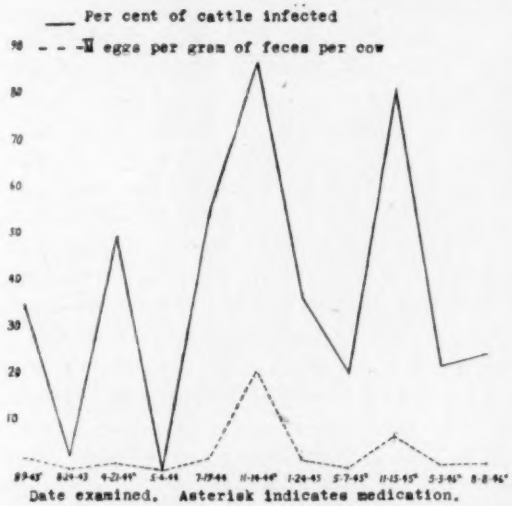
TOLERANCE OF CATTLE FOR HEXACHLOROETHANE

Hexachloroethane has been used as a fasciolicide with relative safety in this country and elsewhere. In the region where

TABLE 6—Weight Gains in 20 Untreated 3- to 4-Year-Old Hereford Cows and Their Calves, and 20 Treated Cows and Calves of Same Age Over a Period of 205 Days. (Medication was with 200 cc. hexochloroethane suspension at the beginning of the observation and again about two months later, at which time the calves received 100 cc.)

CATTLE	AVERAGES PER HEAD				
	INITIAL WEIGHT MAR. 13 (LB.)	FINAL WEIGHT OCT. 3 (LB.)	GAIN 205 DAYS (LB.)	DAILY GAIN	
				LB.	% OF ORIGINAL GAIN
20 untreated Hereford cows	754	928	174	0.85	0.11
20 treated Hereford cows	743	875	132	0.64	0.09
20 untreated Hereford calves	165	479	314	1.53	0.93
20 treated Hereford calves	160	458	298	1.45	0.91

the work reported in this paper was done and where mostly range cattle have been medicated, the animals generally have shown a high tolerance for the suspension, even when given in excessive doses. The clinical reactions occasionally observed in them were a temporary loss of appetite and mild diarrhea. Weak animals sometimes ex-



Graph 4—Fluctuation of incidence of infection with Fasciola hepatica and average egg counts in Hereford cattle on prairie pasture, Refugio County, Texas, during regimen of medication with hexachloroethane suspension semiannually for three years.

hibited dizziness, reeling, drowsiness, and lack of appetite. Some animals were unable to rise from a recumbent position, while others appeared to be in a state of narcotization. This condition usually passed in a few hours. However, some animals failed to rally, and died. The losses from death were very few in view of the large number of cattle that have been treated. The reason for this susceptibility to hexachloroethane by some individuals is unknown.

Microscopic examination of liver and kidney tissues from animals whose deaths were associated with medication with hexachloroethane suspension did not reveal serious damage. In the kidneys, the proximal convoluted tubules showed dilatation, together with some degeneration and desquamation of the epithelial lining in the distal portions. There was no evidence of direct toxic effects in the liver, such as necrosis or pronounced fatty change of the parenchyma.

In Germany, only mild injury, such as occasional slight enlargement of the central veins of the liver lobules together with hyperemia, and nuclear cloudiness of the convoluted tubules of the kidneys, were reported for cattle medicated with Neoserapis.[44] Symptoms following treatment with Neoserapis were stated to be brief loss of appetite, intoxication, reeling, temporary prostration, and occasional deaths.[14,44] Attempts to obviate these untoward effects included mixing the medicant with a suspension of flour and water.[14, 46]

In Germany, chemically pure crystalline hexachloroethane given in gelatin capsules was reported to be well tolerated by cattle even when given in doses of twice the required size.[27] In Hawaii, the use of large doses of unadulterated hexachloroethane caused colic in dairy cows, but was well tolerated when mixed with kamala extract and given on two consecutive days.[1]

The addition of an adulterant appeared to permit the use of larger doses of hexachloroethane without serious effects on the host. The proprietary Igitol, compounded from hexachloroethane and starch compressed into boluses,[38] could be given in a single dose without untoward results. In order to retard absorption and thereby avoid serious after effects, it was recommended that hexachloroethane be mixed with a light adsorbent such as talc.[12]

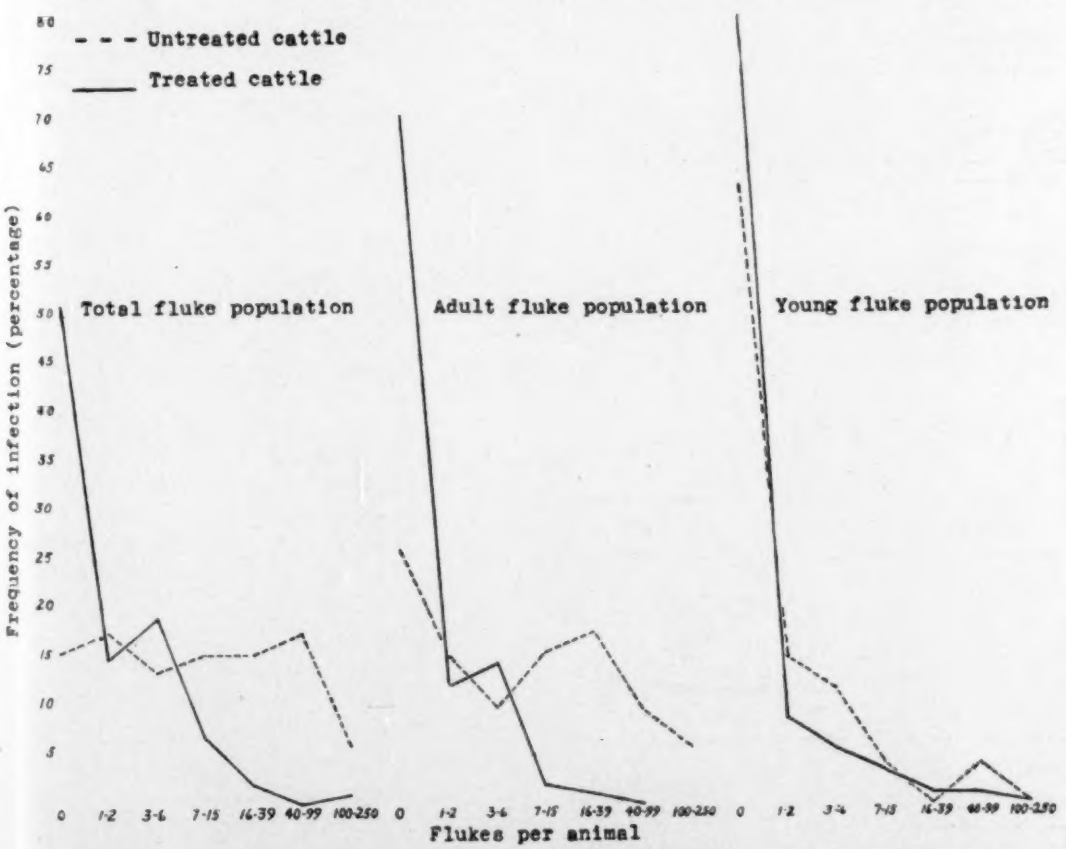
The hexachloroethane-bentonite suspension was developed with the foregoing experiences in mind. The bentonite, a colloidal hydrated aluminum silicate (clay), was selected to serve both as the suspending and adsorbing agent. Medication with the aqueous suspension obviated to a large extent the unfavorable results reported for earlier preparations, and, at the same time, facilitated treatment because the entire dose could be given quickly as a drench.

DISCUSSION

Hexachloroethane suspension is both effective and relatively safe for removing liver flukes from range cattle. Limitations of the drug appear to be associated with (1) extensive pathologic changes in the liver and (2) the resistance of young flukes to the medicament.

Pathologic changes occurring as a result of fascioliasis may greatly impair or completely destroy the physiologic functions of affected portions of the liver. Morrill and Shaw[24] observed, "mechanical destruction of hepatic cells, sinusoids, biliary canaliculi, and related tissue. Adjacent structures also showed the effects of coagulation necrosis, subsequent phagocytosis, and their replacement with connective tissue." They also noted a complete absence of parenchymatous tissue and its replacement by extensive fibrosis in the ventral lobes of severely affected livers. Obstructive biliary cirrhosis may involve the entire choleic system, resulting in increased connective tissue in the portal areas. Under these conditions, the canaliculi become so distended with inspissated bile that associated liver cells may disintegrate, and the canaliculi, in turn, rupture.[5]

In order to be effective, the medicament must reach the flukes through the bile. If the mechanism for its transfer from the blood stream to the biliary system is destroyed, this action cannot be effected. Often the entire terminal portion of the ventral lobe becomes extensively sclerotized and the hypertrophied bile ducts dilated by the accumulation of calcareous deposits resulting from the activities of flukes (fig. 1). Flukes surviving medication are found commonly in such places. It is probable, therefore, that a correlation exists between normal func-



Graph 5—Comparison of the number of liver flukes found at postmortem examination in 52 untreated cattle with the number in 91 cattle treated with hexachloroethane suspension.

tioning of the liver and efficacy of the fasciolicide, which in this case was hexachloroethane. This point of view is further substantiated by the complete destruction of all the flukes in young calves whose livers were either without macroscopic evidence of tissue changes or with only slight changes.

Young migrating flukes, because of their location in the body cavity or liver parenchyma, are not affected by the low concentration of the drug in the blood.[20] Small flukes in the bile ducts are relatively refractory to medication.

Attempts to eradicate liver flukes from areas by semiannual medication of all the cattle on them were not successful. While the fluke population was reduced greatly by medication, a high incidence of infection usually occurred before the next treatment. Three principal factors probably contributed to the persistence of the infection: (1) survival of a few adult flukes, and resistance of young flukes to medication, (2) an abundance of the snail intermediate host on the pastures, and (3) the presence on the pastures of large numbers of wild rabbits in which a high incidence of infection was present. What part small rodents and other animals commonly found on the pastures may have as important reservoir hosts is unknown. The common liver fluke has been found in horses,[3, 15, 25] mules, [9] and pigs.[15, 16] White-footed mice have been infected experimentally with *Fasciola hepatica*.[21] Although deer are found commonly throughout the coastal prairie of Texas, none of the livers examined by the writer have been infected with *F. hepatica*.

In Gulf Coast region where the metacercariae of *F. hepatica* were destroyed by the heat and drought during the summer and fall, the flukes in the cattle generally were mature before the pastures became reinfested[34,36] when winter rains activated the snails.[31] Medication late in the fall before the onset of wet weather destroyed most of the flukes. This condition was shown by both the lower incidence of infection and mean number of eggs per gram of feces at the time of the spring drenching in the two herds of cattle medicated periodically for three years (tables 2, 3; graphs 3, 4). For this reason, some ranchers drench a third time during the year, generally in August.

Since some adult flukes survived the single treatment (table 4), as stated above, two treatments on consecutive days would doubtless be more effective in destroying them, as suggested by Alicata.[2] The added labor of handling large herds of cattle, together with the current high cost of the medicament, discourages this practice.

While medication proved eminently successful in both preventing and alleviating clinical fascioliasis, it did not preclude reinfection when administered semiannually to all the cattle. The total absence of flukes in about 40 per cent more adult livers in the treated than untreated cattle, as shown by postmortem examination (table 4), following one medication, is indicative of the potentialities of a sound control program. It is the opinion of some workers[12,39] that while medication is effective in preventing serious losses among livestock from liver fluke infection, best results are obtained when an effort is directed toward reducing both the fluke and snail populations on the pastures.

SUMMARY

1) Hexachloroethane-bentonite-water suspension proved to be an efficient and relatively safe fasciolicide for use in cattle when administered as a single dose at the rate of 200 cc. for grown cattle and 100 cc. for yearlings.

2) Medication of 463 mature cattle positive for liver flukes resulted in only 35 showing eggs in their feces when examined two to three weeks later. Premedication egg counts averaged 26.4 ± 40.4 (standard deviation) per gram of feces and postmedication counts 4.9 ± 5.7 (s. d.).

3) Dilution egg counts showed approximately 90 per cent of the infected cattle with 50 or fewer eggs per gram of feces, leaving only a small percentage with counts sufficiently high to indicate the possibility of early clinical fascioliasis.

4) Semiannual medication for three years of two herds of cattle kept on their respective pastures prevented clinical fas-

cioliasis but did not preclude recurrence of infection, with great fluctuation in the incidence.

5) The incidence of infection always dropped sharply following each medication and generally increased during the six-month intervals between treatments.

6) Factors probably responsible for recurrence of high infection in these areas were (1) a residue of flukes remaining in the cattle after medication, (2) an abundance of the snail intermediate host on the pasture, and (3) the presence of a reservoir host on the pasture.

7) Wild rabbits, particularly jack rabbits, were found to be heavily infected with liver flukes. Approximately 33 per cent of 178 jack rabbits were infected.

8) Postmortem examination of adult cattle showed 56 per cent of the medicated group free of flukes as compared with 15 per cent of the unmedicated group. The mean number of flukes was 23.9 ± 23.9 (s. d.) for the unmedicated and 3.3 ± 10.6 (s. d.) for the medicated.

9) Immature flukes are refractory to medication with hexachloroethane, due partly to their location in the body during migration.

10) Greatest survival of flukes after medication was found in sections of livers having undergone extensive pathologic change due to fascioliasis.

11) All flukes in young calves with normal or nearly normal livers were destroyed by a single medication, indicating a correlation between normal liver function and fasciolicidal efficacy of hexachloroethane.

12) Weight studies in a limited experiment indicated that under favorable nutritional and weather conditions, such as these cattle experienced, gains in weight did not follow medication with hexachloroethane of cows lightly infected with flukes, nor their calves, over gains in similarly infected but unmedicated cows and their calves.

13) In cases of clinical fascioliasis, medication with hexachloroethane resulted in rapid recoveries and pronounced improvement in physical condition.

14) Range cattle in average condition have a high tolerance for hexachloroethane; debilitated animals may respond unfavorably, or die, from its effects and should be treated with caution.

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Effects of Penicillin on Brucella Suis in Vitro and in Vivo

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Introduction and Review of Literature

The work reported in this paper was originally undertaken in an effort to determine the effectiveness of penicillin as a bacteriostatic agent in controlling contamination found when culturing, on laboratory mediums, naturally infected material for *Brucella suis*. However, preliminary studies indicated that *Br. suis* was inhibited by rather low concentrations of penicillin in the mediums. For this reason, it was decided to investigate further the antibiotic effect of penicillin against the *Br. suis* organism *in vitro* and *in vivo*.

T'ung[1] reported work showing some antibacterial action of penicillin on eight of 15 strains of Brucella *in vitro*. He observed that

TABLE I—Inhibitory Effect of Penicillin in Agar Mediums on Growth of Brucella Suis, First Trial

O.U. Penicillin /cc. Agar	Strain Number						
	1	2	3	5	11	12	37
400	-	-	-	-	0	0	-
200	+	-	0	0	0	0	+
100	-	+	+	-	++	0	+
50	+	+	++	+	+	+	0
25	+	+	0	0	0	0	+
12.5	+	0	++	++	0	0	++
6.25	+	+	0	0	0	0	+++
3.13	+++	+++	++++	+++	0	0	+++
1.56	++++	++++	++++	+++	+++	++++	0
Control	++++	++++	++++	++++	++++	+++	++++

0=No plates available. -=No growth. +=Slight growth at initial point of inoculation only. ++=Small streak of growth at initial point of inoculation. +++=Scanty growth throughout plate. ++++=Heavy growth throughout plate. O.U.=Oxford unit.

Br. suis appeared to be more penicillin-susceptible than did *Brucella melitensis;* however, because of the small number of cultures in each species represented, no conclusions were made.

Schwartzman[2] reported that several highly refractory gram-negative organisms, including Brucella, showed a greater susceptibility to penicillin when methionine, threonine, and methionine sulfoxide were added to broth cultures of the organisms.

The greatest difficulty encountered during the course of this experiment was the apparent toxicity of penicillin for the guinea pig. The observations in this regard in this laboratory were similar to those of other workers. Heilman and Herrell[3] reported: "Young guinea pigs receiving 1,000 to 5,000 units of penicillin daily in divided doses frequently died after several days." Hamre, *et al.*[4] observed that guinea pigs injected with penicillin showed toxic reactions, loss of weight, and death.

The general bacteriologic methods used for this study of the Brucella organisms and for the testing of infected animals are those described by Hutchings[5] and Hutchings, Delez, and Donham.[6]

In Vitro Studies, Materials, and Methods

In order to determine whether penicillin added to culture mediums would have an inhibitory effect on *Br. suis*, 2,500 cc. of bactotryptose agar (Difco) was prepared and divided into ten equal portions. After the agar had cooled to approximately 50 C., 20-, 10-, 5-, 2.5-, 1.25-, 0.625-, 0.313- and 0.156-cc. amounts of a physiologic saline solution of penicillin containing 5,000 Oxford units per cubic centimeter were added to eight of the portions of agar. The remaining 500 cc. of agar was used for control plates. The penicillin agar preparations contained 400, 200, 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 units of penicillin per cubic centimeter, respectively.

Streak cultures were made by streaking a small loopful of viable *Br. suis* cells on these agar plates. The results of such inoculation of plates with seven different strains of *Br. suis* are shown in table 1.

The results obtained in the first trial, considerable inhibition at a concentration of 6.25 units of penicillin per cubic centimeter and above (table 1), suggested a second experiment in which the concentrations of penicillin would be much lower. The second lot of agar was prepared, divided, and portions containing final concentrations of 5, 2.5, 1.3, 0.63, 0.31, 0.16, 0.078, 0.039, 0.020, 0.010, 0.005, 0.002, and 0.001 units of penicillin per cubic centimeter of mediums were made. Such concentrations were prepared by serially diluting 1 cc. of a physiologic saline solution of peni-

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All penicillin used in this study was Penicillin (Sodium Salt), supplied gratis by Eli Lilly and Company, Indianapolis, Ind.

cillin containing 5,000 units. The proper dilutions were added to each of 13 portions of 500-cc. amounts of agar to give the desired number of units of penicillin per cubic centimeter. Thus this dilution system resulted in the amount of added liquid being uniform, regardless of the concentration of penicillin. The results of culturing six different strains of *Br. suis* on these types of mediums are shown in table 2.

TABLE 2—Inhibitory Effect of Penicillin in Agar Mediums on Growth of Brucella Suis, Second Trial

O.U. Penicillin /cc. Agar	Strain Number					
	1	2	3	5	11	12
5	+	+	+	+	+	-
2.5	+	+	+	+	+	-
1.3	++	-	+++	+	+	++
0.63	++	++	+++	++	+	+++
0.31	+++	+++	++++	++	++	+++
0.16	+++	+++	++++	++++	+++	++++
0.078	++++	++++	++++	++++	+++	++++
0.039	++++	++++	++++	++++	++++	++++
0.020	++++	++++	++++	++++	++++	++++
0.010	++++	++++	++++	++++	++++	++++
0.005	++++	++++	++++	++++	++++	+++
0.002	++++	++++	++++	++++	++++	++++
0.001	++++	++++	++++	++++	++++	+++
Control	++++	++++	++++	++++	++++	+++

-=No growth. +=Slight growth at initial point of inoculation. ++=Small streak of growth at initial point of inoculation. +++=Scanty growth throughout plate. ++++=Heavy growth throughout plate. O.U.=Oxford unit.

Since from the above two experiments, it appeared that there was considerable inhibition of growth at a concentration of 6.25 units of penicillin per cubic centimeter of agar mediums, a third trial was conducted to determine quantitatively the inhibitory action of penicillin. Varied numbers of *Br. suis* were cultivated on agar plates containing varied concentrations of penicillin as follows: 10, 5, 2.5, 1.3, 0.625, 0.313, and 0.156 units of penicillin per cubic centimeter of agar. The agar plates were inoculated with 1-cc. amounts of physiologic saline suspensions of 18 different strains of Brucella prepared by adjusting saline suspensions of cells to approximately the density of tube 1 of the McFarland nephelometer,[7] and diluting until they resulted in dilutions of 1:100,000,000 for strains 1 and 3 and 1:10,000,000 for the remainder of the strains. Twenty-four-hour agar slant cultures were used in preparing the cell suspensions. Results are shown in table 3.

Results of In Vitro Studies

Table 1 shows the results of streaking agar plates, containing concentrations of penicillin varying from 400 to 1.56 units of penicillin per cubic centimeter of agar, with seven different strains of *Br. suis*. All five strains tested were completely inhibited when the medium contained 400 units of penicillin per cubic centimeter. One of three strains was completely inhibited at 200 units per cubic centimeter. Inhibition was marked when the medium contained as little as 6.25 units of penicillin per cubic centimeter of agar.

The results of inoculating, in the same manner, plates containing from 5 to 0.001 units of penicillin per cubic centimeter of agar are shown in table 2. The indications from this trial are that there was considerable inhibition of this rather massive inoculation at a concentration of 1.3 units per cubic centimeter of agar, with strain variations in sensitivity. The variation of inhibition between these two trials (inhibition at 6.25 units of penicillin/cc. concentration in the first trial, and at a concentration of 1.3 units of penicillin/cc. in the second) could be explained by the fact that there is commonly much variability in growth of *Br. suis* on laboratory mediums. Also, these first two trials were of an exploratory nature, and because of the manner of inoculation there was possibility of variation in the amount of inoculum, although an effort was made to keep the amount as constant as possible.

Table 3 presents the data resulting from the inoculation, in duplicate, of plates containing from 10 to 0.156 units of penicillin per cubic centimeter of agar with 1-cc. amounts of a 1:10,000,000 dilution of suspensions of the approximate density of tube 1 of the McFarland nephelometer,[7] of 15 strains of *Br. suis*. One-cc. amounts of 1:100,000,000 dilutions of strains 1 and 3 were used for inoculation. A 1:10,000,000 dilution of strain 18, a *Br. abortus* strain which grows aërobically, was used.

In this trial, three strains of *Br. suis* (3, 5, and 11) were completely inhibited at a concentration of 0.313 unit of penicillin per cubic centimeter of medium. Five additional strains of *Br. suis* (12, 13, 14, 15, and 16) and the one strain of *Br. abortus* (18) were completely inhibited at a concentration of 0.625 unit of penicillin per cubic centimeter of agar. *Br. suis* strains 1, 2, and 6 were completely inhibited at a concentration of

1.3 units of penicillin per cubic centimeter of agar. All but two strains (7 and 9) were completely inhibited at a concentration of 5 units of penicillin per cubic centimeter of agar. Strain 9 was the only strain which showed growth at a concentration of 10 units of penicillin per cubic centimeter of medium. Thus, these data show marked differences in strain sensitivity of Brucella organisms to the *in vitro* antibiotic activity of penicillin.

IN VIVO STUDIES, MATERIALS AND METHODS

In order to test the suspected toxicity of penicillin for guinea pigs, a preliminary study was made using 14 guinea pigs. Five groups of 2 guinea pigs each received daily injections of 210, 420, 840, 1,680, and 3,360 units of penicillin. The 4 remaining guinea pigs were kept as controls. The penicillin was injected in physiologic saline solution, intramuscularly.

The 2 guinea pigs receiving the highest dose of penicillin died. Sections of kidney, lung, liver, spleen, and adrenal were saved for histologic examination.

The 12 guinea pigs which survived were observed closely for ten days after the final injection of penicillin and were used again in the major *in vivo* experiment. Care was exercised in distributing these animals among the groups in order not to bias the results of that study.

In order to determine the *in vivo* effect of penicillin in combating brucellosis, a factorially designed experiment was employed using a total of 180 guinea pigs. The guinea pigs were divided into 36 groups of 5 animals each. Three groups received the low exposure dose (74 organisms), three groups received the medium exposure dose (740 organisms), and three the high exposure dose (7,400 organisms) of *Br. suis*. In order to determine the number of organisms in the inoculum used in exposing the guinea pigs, a suspension of a mixture of four strains of *Br. suis* in physiologic saline was standardized to a density corresponding to tube 1 of the McFarland nephelometer.[7] The suspension was diluted in physiologic saline to 1:100,000, 1:1,000,000, and 1:10,000,000. One cubic centimeter of the final dilution was plated in triplicate. The average plate count was 74 organisms per cubic centimeter of the 1:10,000,000 dilution.

TABLE 3—Inhibitory Effect of Penicillin in Agar Mediums on Growth of Brucella Suis, Third Trial

Strain	Control	Oxford Units of Penicillin /cc. of Agar						
		0.156	0.313	0.625	1.3	2.5	5.0	10.0
1* C	5.57	5	5.5	1	.....	.....	.....	.....
%	100.00	87.72	98.74	17.95	.....	.....	.....	.....
2 C	20.57	0	0	1	.....	.....	.....	.....
%	100.00	0	0	4.86	.....	.....	.....	.....
3* C	5.75	2	.....	.....	.....	.....	.....	.....
%	100.00	34.78	.....	.....	.....	.....	.....	.....
5 C	47.29	55	.....	.....	.....	.....	.....	.....
%	100.00	116.30	.....	.....	.....	.....	.....	.....
6 C	62.57	63.50	24	.5	.....	.....	.....	.....
%	100.00	101.48	38.36	.8	.....	.....	.....	.....
7 C	50.57	61	51	6	.5	.5	.5	.....
%	100.00	120.62	100.85	11.86	.99	.99	.99	.....
8 C	41.00	38.5	1	.5	.....	.5	.....	.....
%	100.00	93.90	2.4	1.2	.....	1.2	.....	.....
9 C	30.50	35	23	16.5	15.5	1	1	.5
%	100.00	114.75	75.41	54.10	50.82	3.2	3.2	1.6
10 C	38.14	35	11.5	5	1	.5	.....	.....
%	100.00	91.77	30.15	13.11	2.6	1.3	.....	.....
11 C	40.57	3.5	.....	.....	.....	.....	.....	.....
%	100.00	.86	.....	.....	.....	.....	.....	.....
12 C	78.00	24	1.5	.....	.....	.....	.....	.....
%	100.00	30.77	1.92	.....	.....	.....	.....	.....
13 C	69.57	36	.5	.....	.....	.....	.....	.....
%	100.00	51.75	.70	.....	.....	.....	.....	.....
14 C	28.00	22.5	7.5	.....	.....	.....	.....	.....
%	100.00	80.36	26.79	.....	.....	.....	.....	.....
15 C	49.00	41.5	21.5	.....	.....	.....	.....	.....
%	100.00	84.69	43.88	.....	.....	.....	.....	.....
16 C	30.00	24	7	.....	.....	.....	.....	.....
%	100.00	80.00	23.33	.....	.....	.....	.....	.....
17 C	25.00	24.5	22.5	20	7	.....	.....	.....
%	100.00	98.00	90.00	80.00	28.00	.....	.....	.....
18† C	70.00	65.5	21.5	.....	.....	.....	.....	.....
%	100.00	93.57	30.71	.....	.....	.....	.....	.....

0=No plates available. . .=No growth. C=The average number of colonies on duplicate plates or on seven plates in the case of controls. % is based on the supposition that the growth on the control plates was 100 per cent.

*The plates for strains 1 and 3 were inoculated with 1 cc. amounts of 1:100,000,000 dilutions of cell suspensions, while the remainder of the plates were inoculated with 1 cc. amounts of 1:10,000,000 dilutions of cell suspensions.

†Strain 18 is an aerobic strain of *Br. abortus*.

The guinea pigs were injected subcutaneously with the above three dilutions, thus receiving approximately 74, 740, and 7,400 organisms each. The four strains used for the inoculum had previously been demonstrated to be pathogenic for the guinea pig. These three groups did not receive penicillin treatment and served as controls.

The influence of numbers of organisms and amount and time of penicillin treatment were studied in 27 groups of 5 guinea pigs each. All possible combinations of treatment were employed. The numbers of organisms used were approximately 74, 740, and 7,400. Penicillin was administered at levels of 800, 1,600, and 3,200 Oxford units in eight divided doses daily. Some guinea pigs received penicillin at the time of exposure and for four subsequent days. In others, penicillin treatment was carried out from the eighth to the twelfth day and, in others, from the fifteenth to the nineteenth day.

The penicillin solution was prepared for administration by dissolving the contents of a vial containing 100,000 units of penicillin in 20 cc. of distilled water. The 20 cc. was diluted to 200 cc. in distilled water; thus each cubic centimeter contained 500 units of penicillin. Injections were made intramuscularly and subcutaneously, alternately, every three hours for a period of five days. These different inoculation sites were used in an effort to minimize the local tissue reaction due to the injections.

Guinea pigs which died during the course of the experiment were subjected to autopsy, livers and spleens were cultured for *Br. suis*, and tissues of liver, kidney, cecum, and adrenal were saved for histopathologic examinations. Four weeks after exposure, all of the remaining guinea pigs were sacrificed and similar examinations were made. Standard agglutination tests were conducted with the serum of each guinea pig in the following dilutions: 1:25, 1:50, 1:100, 1:200, and 1:400. Tables 4, 5, 6, and 7 show the data accumulated from these studies.

TABLE 4—Results of Exposing 45 Guinea Pigs to Various Numbers of Brucella Suis Organisms

No. *Br. suis* Organisms	No. Guinea Pigs in Group	No. Infected		No. Guinea Pigs Not Infected	No. Guinea Pigs Dead Prior to Autopsy
		Pos. Agg.	Pos. Culture		
74	15*	7	7	7	0
740	15*	14	14	0	0
7,400	15*	13	12	1	0
Totals	45	34	33	8	0

*One guinea pig from each group was killed one week after exposure to see whether infection could be detected at this time.

Results of in Vivo Studies

The 2 animals receiving the highest dose of penicillin in the preliminary toxicity experiments died, 1 about three hours after receiving the last injection, the second within twelve hours after the last injection. The 10 guinea pigs in the other five groups of this experiment showed ruffled coats, loss of weight, anorexia, and general apathy in varying degrees. The 4 controls remained normal. Postmortem examinations revealed little. The areas around the injection sites were edematous and hemorrhagic. A general atony of the gastrointestinal tract was observed, which may have been due to the general debility of the guinea pigs. His-

TABLE 5—Results of Initiating Penicillin Treatment of Guinea Pigs at the Time of Exposure to Brucella Suis

No. *Br. suis* Organisms	Daily U. Penicillin for 5 Days	No. Guinea Pigs in Group	No. Infected		No. Guinea Pigs Not Infected	No. Guinea Pigs Dead Before Autopsy
			Pos. Agg.	Pos. Culture		
74	800	5	3	3	0	2
740	800	5	2	2	0	3
7,400	800	5	4	4	0	1
74	1,600	5	2	2	1	2
740	1,600	5	4	4	0	1
7,400	1,600	5	2	2	0	3
74	3,200	5	4	5	0	0
740	3,200	5	3	3	0	2
7,400	3,200	5	3	3	0	2
Totals		45	27	28	1	16

tologic examination of the tissues showed no lesions which could be attributed to penicillin administration. The kidneys showed a moderate degree of parenchymatous degeneration which could be the result of a toxic reaction.

Table 4 shows the effect of exposing guinea pigs to three different doses of a polyvalent suspension of *Br. suis*. One guinea pig from each exposure level was killed one week after exposure to determine if infection could be detected at that time. The bacteriologic findings and agglutinin responses of these 3 animals were negative for Brucella infection. Of the remaining 14 guinea pigs exposed to 74 organisms, 7 (50%) were shown to be infected at the time of autopsy by the criteria employed, the isolation of *Br. suis* from either liver or spleen and/or a positive agglutination reaction at 1:25 or higher dilution of the serum. All of 14 remaining guinea pigs were found to be infected when exposed to 740 organisms. Of the 14 guinea pigs exposed to 7,400 organisms, 13 (93%) were infected. None of the control guinea pigs died before the four-week period of the experiment was completed.

The results of initiating treatment one week after exposure are given in table 6. Fourteen (31%) of all animals receiving this treatment, regardless of Brucella exposure dose, died prior to the end of the experimental period of four weeks. Five (16%) of those which survived were found not to be infected. Of those 5, 2 (17%) were from the 12 surviving exposed to the low dose of Brucella, and 3 (33%) were from the 10 surviving exposed to the medium dose of the organism. All of the surviving guinea pigs exposed to 7,400 organisms were infected at the time of sacrifice.

Table 7 shows the results when treatment was initiated two weeks after exposure. Nineteen (42%) of 45 died prior to sacrifice. Three (12%) of the remaining 26 guinea pigs were found not to be infected. These were all from the low Brucella exposure group.

Seventeen (38%) of the 45 guinea pigs receiving the low dose of penicillin, 17 (38%) of 45 receiving the medium dose of penicillin, and 15 (33%) of the 45 receiving the high dose of penicillin, regardless of the Brucella exposure dose, or the time of initiation of treatment, died prior to the time of sacrifice. This was a total of 49 (36%) of 135 guinea pigs receiving penicillin treatment which died during the course of the experiment.

TABLE 6—Results of Initiating Penicillin Treatment of Guinea Pigs One Week After Exposure to Brucella Suis

No. *Br. suis* Organisms	Daily U. Penicillin for 5 Days	No. Guinea Pigs in Group	No. Infected		No. Guinea Pigs Not Infected	No. Guinea Pigs Dead Before Autopsy
			Pos. Agg.	Pos. Culture		
74	800	5	3	3	1	1
740	800	5	1	1	2	2
7,400	800	5	2	2	0	3
74	1,600	5	2	2	1	2
740	1,600	5	4	4	0	1
7,400	1,600	5	2	2	0	3
74	3,200	5	5	5	0	0
740	3,200	5	2	2	1	2
7,400	3,200	5	5	5	0	0
Totals		45	26	26	5	14

Table 5 presents the results of treating guinea pigs, exposed to three levels of *Br. suis* with three different daily amounts of penicillin, treatment initiated at the time of exposure. Sixteen (36%) of the 45 guinea pigs, regardless of Brucella exposure dose, died during the period of treatment. Only 1 (3%) of the remaining 29 guinea pigs was not infected at the time of sacrifice.

Postmortem examination of the guinea pigs which died prior to sacrifice generally revealed an enlarged cecum filled with very watery contents. There was injection of mesenteric blood vessels, inflammation of the cecum in most cases and, in some cases,

of the stomach wall. Slight hemorrhage and grayish white foci were apparent from the serous side of the cecum. The mortality was greatest from approximately seventy-two hours after the initiation of treatment until the end of the five-day injection period. Few guinea pigs died after penicillin injections were stopped.

Histologic examination of the hearts, livers, spleens, and adrenals revealed no lesions which could be attributed to penicillin injections. Kidneys examined showed moderate parenchymatous degeneration, but nothing severe enough to have been the cause of death in the animals. There was considerable desquamation and disorganization of the cecal epithelium, some of which could have been due to postmortem decomposition, and some necrosis. The lymph follicles of the cecums were edematous, probably the cause of the appearance of the grayish white foci which were observed from the serous surface of the cecum.

The guinea pigs which survived the penicillin treatment showed no gross lesions at the time of sacrifice except those usually found in Brucella-infected individuals.

TABLE 7—Results of Initiating Penicillin Treatment of Guinea Pigs Two Weeks After Exposure to Brucella Suis

No. *Br. suis* Organisms	Daily U. Penicillin for 5 Days	No. Guinea Pigs in Group	No. Infected		No. Guinea Pigs Not Infected	No. Guinea Pigs Dead Before Autopsy
			Pos. Agg.	Pos. Culture		
74	800	5	3	3	2	0
740	800	5	4	4	0	1
7,400	800	5	1	1	0	4
74	1,600	5	1	1	1	3
740	1,600	5	3	3	0	2
7,400	1,600	5	5	4	0	0
74	3,200	5	3*	4	0	1
740	3,200	5	1	1	0	4
7,400	3,200	5	1	1	0	4
Totals		45	22	22	3	19

*A serum sample was not obtained from 1 guinea pig.

Discussion

The objective of these studies was twofold: (1) to find at what concentration of penicillin in artificial mediums *Br. suis* was inhibited and (2) to study the effect of administration of various amounts of penicillin, initiated at different periods of time in the course of infection on brucellosis produced by experimental infection of the guinea pig.

With a comparatively massive inoculation, *Br. suis* was greatly inhibited at a concentration of 6.25 Oxford units of penicillin per cubic centimeter of agar medium. When smaller inoculations of medium were made to determine quantitatively the amount of inhibition, 0.625 unit of penicillin per cubic centimeter of agar caused inhibition in all 17 of the strains tested, with strain variations. Eight strains of *Br. suis* and the one strain of *Br. abortus*, growing aërobically, were completely inhibited at this level, while the remaining eight strains of *Br. suis* showed 50 per cent or more inhibition. Strain differences in sensitivity were emphasized by the observation that, while all of the strains were inhibited at a low concentration of penicillin, growth of two strains persisted at a concentration of 5 units of penicillin per cubic centimeter of agar and one showed growth at the level of 10 units per cubic centimeter of medium.

Although all guinea pigs receiving the low exposure to *Br. suis* did not appear to be infected at autopsy, the response was not significantly different from that of the control group in so far as the per cent of infection produced. There was no recovery from the infection that could be attributed to the penicillin treatment in any case.

The apparent toxic reactions produced in guinea pigs by the penicillin are of interest. The results of the preliminary toxicity study suggested that there would be a direct

relationship between the amount of penicillin injected and the toxic reaction produced. In the major experiment there was no apparent relationship between the number of units of penicillin administered and the number of guinea pigs which died, supposedly due to penicillin treatment. It would have been well to inject at least part of the control animals with a physiologic saline solution for a comparable period of time to see whether such treatment would cause similar injurious effects. This possibility was overlooked at the time the experiment was planned. It is conceivable that frequent handling and hypodermic injection of guinea pigs could cause a reaction. However, it seems unlikely that such would be severe enough to result in death in such a high percentage of cases.

The histopathologic findings were moderate. The only conditions found in the tissues which were examined that could be attributed to a possible toxic reaction were the slight parenchymatous degenerative changes in the kidneys and the cecal lesions —edematous lymph follicles and the desquamation and necrosis of the epithelium.

SUMMARY

1) With massive inoculation, *Brucella suis* was inhibited when concentrations of penicillin of 6.25 units per cubic centimeter were added to bacto-tryptose agar mediums, with strain variations in sensitivity.

2) When smaller inoculations were made, marked inhibition was apparent at a concentration of 0.625 unit of penicillin per cubic centimeter of bacto-tryptose agar mediums, with strain variations.

3) Treatment of *Br. suis*-infected guinea pigs with penicillin was not effective in combating brucellosis as tested in these studies. Varying exposure doses of *Br. suis* and varying levels of penicillin with treatment initiated at different times after exposure had no significant effect in altering the course of the disease.

4) Administration of penicillin to guinea pigs apparently resulted in death in 36 per cent of the 135 animals during treatment, regardless of the size of the daily dose of penicillin from 800 to 3,200 units. The guinea pigs showed ruffled fur, anorexia, loss of weight, and were generally apathetic prior to death. No deaths occurred in the control guinea pigs during the period of the experiments, but unfortunately they were not handled and injected every three hours comparable to such treatment to which the principal guinea pigs were subjected.

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The Immunization of Guinea Pigs with Mucoid Phases of Brucella

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THE WRITER has described[1] three different mucoid (M) phases arising from smooth cultures of *Brucella abortus*, two from *Brucella suis*, and one from *Brucella melitensis*. Continued studies in this direction revealed two additional M phases arising from *Br. suis* and one from *Br. melitensis*. One of those obtained from *Br. suis* gave rise to daughter colonies that resembled in all respects the smooth (S) phase. Cells of these colonies when suspended in saline solution were agglutinated by S antiserums and were pathogenic for the guinea pig. The new M phase from *Br. melitensis* also gave rise to numerous S-like daughter colonies, but cells of these in physiologic salt solution agglutinated spontaneously and, furthermore, when injected into guinea pigs did not produce a progressive type of disease.

When live cell suspensions of any of the pure M phase cultures were injected intraperitoneally or subcutaneously into normal guinea pigs in numbers varying from 5×10^9 to 1×10^{10}, a nonprogressive type of infection was produced. Those guinea pigs killed and examined one week after injection showed a fibrinous membrane attached to the liver and omentum. Mucoid organisms were cultured from the spleen, liver, kidney, omentum, and testes. Those pigs sacrificed at the end of two weeks after injection showed only a slight thickening of the omentum. Mucoid organisms were cultured from only the liver and spleen at this time. Three weeks after injection, the spleen was the only organ showing the presence of mucoid organisms. All culture examinations made of the tissues at the end of the fourth week were negative.

Pure M phase cells in a live state do not appear to be capable of producing serum agglutinins in animals in a high titer for an S phase antigen. When rabbits are injected intravenously with 5×10^9 to 1×10^{10} cells, serum agglutination titers of 1:640 to 1:1,280 may be obtained in fifteen days. The subcutaneous injection of cattle with two or three times the maximum number of cells just mentioned rarely raises the serum agglutination titer above 1:100. This usually occurs within fifteen days. The agglutination titer falls below 1:25 within sixty to ninety days after the injection.

The low pathogenicity and the low agglutinin titer response which characterize the M growth phases of Brucella led the writer to initiate experiments to determine their possible value as agents for producing active immunity in experimental animals against brucellosis. The guinea pig was chosen for this purpose, first because of its susceptibility to infection from the injection of a small number of pathogenic Brucella cells, and second, only a short time is required in which to complete an experiment. It may also be said that the guinea pig is one species of animal in which it has been difficult to produce an active immunity against brucellosis.

EXPERIMENTAL METHODS

The different M phase vaccines were prepared from suspensions of the cells after sufficient growth had been obtained in a suitable liquid culture medium.[2] The medium was shaken constantly during the incubation period. Because of the nature of the growth and the presence of large numbers of dead cells, dosage of the vaccine was based on dry weight of cells. The cells are very unstable in suspension and will settle rapidly to the bottom of a container. In the several vaccines studied, the live cell count varied from 5×10^9 to 1×10^9 per milligram of dried cells. These counts represent approximately one-half of the cells present. It will be shown in the experimental

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data that the number of live organisms present in a suitable vaccine is not as important as the preservation of a very labile immunogenic agent contained in the cells.

The first trial dose contained 1 mg. of cells. The selection of this size dose proved to be very fortunate for when smaller amounts (0.5 mg. or less) were tried later, many of the pigs in each group treated failed to resist infection.

All of the experimental trials have been conducted with groups of 10 pigs. The vaccines were injected either subcutaneously or intradermally. The final results were similar regardless of the route of injection. Approximately four weeks after receiving the vaccine, the pigs along with an equal number of controls were injected subcutaneously with live cells of a virulent culture of one of the species of Brucella. When *Br. suis* was used as the challenging species, 1×10^3 cells were given. The challenging dose of *Br. abortus* or *Br. melitensis* contained either 5×10^4 or 1×10^5 live cells. The internal organs were examined for macroscopic evidence of infection and were cultured on tryptose agar for Brucella organisms. The blood serum of each pig at this time was examined for Brucella agglutinins. The final criterion of infection was the isolation of Brucella organisms from the tissues.

EXPERIMENTAL RESULTS

During the past three years, 50 separate experiments have been conducted on groups of 10 guinea pigs with live or inactivated cell suspensions of certain of the M phase cultures, or pure daughter-colony cultures derived from one or more of the M phase cultures.

In those experiments in which immunization was attempted with inactivated organisms, the inactivating agent was either heat, phenol, phenylmercuric nitrate, formalin, or irradiation with ultraviolet light. All agents used, with the exception of 0.25

TABLE I—The Comparative Value of Several Mucoid Brucella Vaccines in Protecting Guinea Pigs against Brucellosis (Summary of 27 Experiments)

No. experiments	Type vaccine 1-mg. dose	Exposure, species, & no. organisms	No. controls / No. vaccinated	No. controls found infected / No. vaccinated found infected
7	suis M, p2	suis 1×10^3	70 / 70	65 / 7
3	suis M, p2	abortus 1×10^5	30 / 30	25 / 3
3	suis M, p2	melitensis 1×10^5	30 / 30	30 / 4
2	suis M-dc.	suis 1×10^3	20 / 20	20 / 7
1	suis M-dc.	melitensis 1×10^5	10 / 10	10 / 0
5	abortus mucoids	suis 1×10^3	50 / 50	50 / 25
2	abortus M, p3	abortus 1×10^5	20 / 20	12 / 3
1	melitensis M, p1	suis 1×10^3	10 / 10	10 / 9
1	melitensis M, p1	melitensis 1×10^5	10 / 10	10 / 5
1	melitensis M, p2	suis 1×10^3	10 / 10	9 / 1
1	melitensis M, p2	melitensis 1×10^5	10 / 10	10 / 2

M = Mucoid. p = phase of mucoid colony. dc = daughter colony of mucoid phase.

per cent formalin and ultraviolet light, also inactivated the immunogenic agent in the cells. In other words, no resistance against infection could be demonstrated after their injection into guinea pigs. Formalin in the concentration mentioned and ultraviolet light reduced, but did not destroy completely, the immunogenic principle.

The results of 27 immunization experiments with M phase vaccines are summarized in table 1. The results of a few of the experiments with a given mucoid vaccine were combined and the results presented together. A study of the data in the table shows clearly that the highest degree of protection against all three species of Brucella was obtained from the injection of either M phase 2 *suis* or M phase 2 *melitensis*. Of the 70 pigs which were treated with M phase 2 *suis* vaccine, 7 showed no resistance to the challenging dose of *Br. suis*. All of the failures did not occur in one experiment. In two of the trials, none of the treated pigs were found infected. In three others, 1 in each became infected. Two failed to resist infection in each of the two remaining trials.

The daughter-colony vaccine obtained from M phase 2 *suis* also produced a high degree of resistance against infection with either *Br. suis* or *Br. melitensis*.

Three different M phase *abortus* vaccines were prepared and injected separately into either one or more of seven groups of guinea pigs. Five of the groups were exposed to *Br. suis* and two to *Br. abortus*. Since approximately one-half of the treated pigs used in each experiment became infected after they were exposed to *Br. suis*, one would conclude that vaccines of this type do not produce a high degree of resistance against infection.

A vaccine prepared from M phase 1 *Br. melitensis* failed to protect guinea pigs against *Br. suis* infection. The same type vaccine protected 5 of 10 pigs against *Br. melitensis* infection. All 10 of the controls showed extensive infection.

The results obtained with a vaccine prepared from M phase 2 *Br. melitensis* indicated that it was capable of producing a high degree of immunity against all three species of Brucella. In the two experiments in which it was used, 9 of the 10 pigs exposed to *Br. suis* and 8 of the 10 exposed to *Br. melitensis* showed no evidence of infection when examined on autopsy. Of the 20 controls inoculated at the same time, 19 showed extensive infection.

A series of experiments was conducted on several groups of guinea pigs for the purpose of determining the immunizing value of M phase 2 *suis* cells after inactivation by three different concentrations of formalin. The final concentration of formalin in each of the three lots of vaccine prepared was 1.0, 0.5, and 0.25 per cent. One-milligram amounts of the first and second lots of treated cells were injected separately into a group of 10 pigs and inoculated thirty days later with *Br. suis*. When killed and examined after a succeeding thirty days, all were found infected. Two groups of 10 pigs injected with the 0.25 per cent formalin-treated vaccine. One group was inoculated with *Br. melitensis* and another with *Br. suis*. After the usual incubation period, autopsy and culture examinations revealed that 5 of those exposed to *Br. melitensis* and 3 of those exposed to *Br. suis* were infected. All of the 10 controls in each group were infected.

Through the assistance of Dr. Sidney O. Levinson and his associates of the Michael Reese Foundation, several experiments were performed to determine the effect of ultraviolet irradiation on the immunizing value of several lots of M phase *suis* cells. The apparatus which was employed has already been described.[3] Its value as a means of inactivating viruses and bacterial vaccines for immunizing purposes has already been explored and reported.[4,5,6]

After several irradiation trials, it was found that one and a half (1.5) seconds of irradiation by this method was required to inactivate all cells in a suspension containing from 4 to 5 x 10^9 live cells and approximately the same number of dead cells per cubic centimeter. Intervals of irradiation between two-tenths (0.2) and one second killed all except an average of 70 cells per cubic centimeter. In other words, the numbers of cells that escaped death remained

approximately the same regardless of the time of exposure between two-tenths (0.2) and one second. The results of the ultraviolet irradiation experiments are summarized in table 2. The first group of pigs recorded in the table were not treated with the irradiated vaccine until after it had been stored at 4 C. for three months. At this time it contained 35 live organisms per milligram of cells (dry wt.). After being treated with the vaccine, the pigs, with 10 normal controls, were inoculated with a virulent culture of *Br. melitensis*. When the treated pigs were killed and examined, none were found infected. The results of this experiment show conclusively that a large number of viable M cells are not required for the production of a high degree of resistance in guinea pigs against Brucella infection. This point has received further confirmation from the results of another similar experiment in which all of the M cells in the lot of vaccine were inactivated by irradiation for one and a half (1.5) seconds. In this experiment the guinea pigs were treated with the vaccine ten days after irradiation. They were exposed to *Br. suis*. On autopsy, only 2 of those vaccinated were found infected. Nine of the 10 controls were infected. A second immunization trial with the same lot was made after it had been stored for three months at 4 C. This trial was a complete failure. No protection was obtained against exposure to either *Br. suis* or *Br. melitensis*. The results of these two experiments tend to indicate that when the cells are irradiated for a long period, the immunogenic factor in the cells is damaged and that the damage is progressive. These results tend to confirm similar experiences of Dr. Levinson and his associates.

A third immunization experiment was conducted with an M cell preparation containing only 1×10^9 cells per cubic centimeter. The irradiation time was three-tenths (0.3) of a second. After treatment, the suspension was concentrated to 2 mg. per cubic centimeter. This lot of vaccine was found to contain 5 live cells per milligram of cells. One week after preparation, 20 pigs were each treated with 1 mg. of cells. After the usual interval, one group of 10 was exposed to *Br. suis* and one to *Br. melitensis*. When killed thirty days after exposure to infection, 4 of those in the

TABLE 2—Results of Attempts to Immunize Guinea Pigs against Brucellosis by Means of Mucoid Phase Cells of Brucella Suis after Irradiation (Ultraviolet Light)

No. experiments	Type cell suspension	Exposure, species, & no. organisms	No. controls / No. vaccinated	No. controls found infected / No. treated found infected
1	suis mucoid*	melitensis 5×10^4	10/10	10/0
1	suis mucoid†	suis 1×10^9	10/10	9/2
1	suis mucoid‡	suis 1×10^9	10/10	10/8
1	suis mucoid‡	melitensis 5×10^4	10/10	10/10
1	suis mucoid§	suis 1×10^9	10/10	10/4
1	suis mucoid§	melitensis 5×10^4	10/10	10/5

*35 live organisms in each dose, 1 mg.
†All cells killed by treatment. Pigs injected ten days after irradiation of cells.
‡Same lot as (b). Pigs injected two months after irradiation of cells.
§10 live organisms in each dose, 1 mg.

group exposed to *Br. suis* and 5 of those in the group exposed to *Br. melitensis* were found infected. All 20 controls were infected. The results of this experiment indicate that irradiation caused a partial reduction in the immunogenic antigen in the bacterial cells.

DISCUSSION

The results of the foregoing experiments show that properly prepared cell suspensions of certain mucoid growth (M) phases of the Brucella group, when injected into guinea pigs, will engender the development of a sufficient degree of immunity to protect them against brucellosis. The immunity produced by either M phase 2 *suis* or M phase 2 *melitensis* is of such a nature that protection is obtained against infection with the heterologous species as well as the homologous one. The finding of a Brucella immunizing agent which possesses such properties is of considerable practical importance in view of the fact that cattle and human beings are now being found infected with all three species and hogs with two of the species. These recent findings would limit the usefulness of any vaccinal agent if it failed to induce sufficient immunity to protect against *Br. suis* and *Br. melitensis*, as well as *Br. abortus*.

It has been a repeated observation in this study that the injection of large numbers of live cells of pure M phases into guinea pigs failed to stimulate specific serum agglutinins for S antigens when the serum was diluted 1:25 or above. The M phases do, however, produce agglutinins for S antigens when injected intravenously into rabbits. The agglutination titers are rarely higher than 1:1,280.

It has also been observed, without a single exception, that when the treated pigs are found free from infection at necropsy, specific agglutinins for S antigens are absent in serum dilutions of 1:25 or above. In other words, the infecting dose of S organisms does not produce agglutinins in guinea pigs that have been actively immunized with the M phase vaccine. The failure to demonstrate serum agglutinins in guinea pigs after injection of the vaccine or infective organisms would indicate that active immunity to brucellosis in these animals is not associated with the production of this type of antibody. There are, however, present in the serums of guinea pigs treated with M phase vaccines, antibodies which in conjunction with serum complement will inhibit the growth of Brucella *in vitro*. Such antibodies may be demonstrated in serum in dilutions up to 1:20,000 in a suitable *in vitro* system. It is possible that active immunity against infection is partly associated with the presence of this type of antibody.

The favorable results obtained with vaccines prepared from certain of the M growth phases of Brucella in protecting guinea pigs against brucellosis are not in themselves sufficient proof that equally satisfactory results will follow when used in other animals or human beings. Explorations of their value in this direction in cattle in privately owned herds have been in progress for the past two years. The results, thus far, have been highly encouraging. They will be reported in the near future.

SUMMARY

When live cell suspensions of certain mucoid growth phases of *Brucella suis* and *Brucella melitensis* were prepared in a suitable concentration and injected into normal guinea pigs, they were capable of engendering a high degree of active immunity against experimental infection with all three species of Brucella.

Cell suspensions of *Br. suis*, M phase 2, after irradiation with ultraviolet light to a sufficient degree to render all but a few cells in a 1-mg. dose nonviable, were also capable of producing a high degree of immunity in guinea pigs against experimental infection with *Br. suis* and *Br. melitensis*.

The vaccine-treated guinea pigs that were found free from infection after exposure did not show specific agglutinins in the blood serum in dilutions of 1:25 or above.

The results of the experiments reported herein suggest the possibility of obtaining a high degree of immunity with this type

of Brucella vaccine against brucellosis in animals and human beings.

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Intracutaneous Brucellosis Tests in Cattle

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SONIC FILTRATE of *Brucella abortus* (SF) has been investigated as a possible allergic skin-test material in the diagnosis of brucellosis in cattle.[1,2] Like other materials used by various investigators in animals and man, SF was found to be agglutinogenic. Therefore, it could not be used in the study of allergic skin sensitivity of entire herds of cattle since animals free of brucellosis would have been made positive to the agglutination test for varying periods of time. Thus, in order to investigate the practical use of an allergic skin test in cattle, it was essential that the material used be nonagglutinogenic.

MATERIALS AND METHODS

According to Seibert,[3] a diminution in the antigenic activity of the protein in old tuberculin is the result of the heat used in its preparation. Therefore, in the search for a nonantigenic skin-test material in brucellosis, the SF of *Br. abortus* was heated, and it was found that if it was boiled at a low pH (approximately pH 3.0) its agglutinogenic quality was markedly reduced while its ability to produce allergic reactions was not impaired. At about the same time it was found that Krasov[4] had described the preparation of a nonantigenic allergic skin-test material for brucellosis. The method he used in the preparation of the skin-test material is briefly as follows: a suspension of *Br. abortus* is washed with alcohol repeatedly, then dried, suspended in 0.2 N sulfuric acid and autoclaved at 120 C. for twenty minutes. After autoclaving, the fluid part is separated and adjusted successively to pH 3.2, 5.3, 6.7, and 7.6, the precipitates being removed as they form at each pH level. Then, after the last precipitate is discarded, the supernatant fluid is mixed with 7 parts of ethyl alcohol, whereupon a precipitate forms which is dried. Finally, the dried powder is dissolved in 0.85 per cent NaCl, and is used as skin-test material. The reason given by the author for removal of the precipitates at the various pH levels was the finding that injections of the individual redissolved precipitates into separate groups of rabbits stimulated the production of low-grade agglutination titers. At first the above mode of preparing the skin-test material was adhered to, but it was soon found that the method could be simplified.

Modified Method of Preparing a Nonagglutinogenic Skin-Test Material.—Large quantities of *Br. abortus* were grown on tryptose agar in flasks for forty-eight hours; then the growth was washed off with 0.85 per cent NaCl. Thereafter it was centrifuged, the supernatant fluid was removed, and the sediment was again washed in 0.85 per cent NaCl. After a second centrifuging, the supernatant fluid was removed and the sediment washed eight times with absolute ethyl alcohol. Finally, it was washed twice with ether and dried by vacuum. The dry material was suspended at the ratio of 1 per cent of dry weight in 0.2 N sulfuric acid and autoclaved at 120 C. for twenty minutes. After autoclaving, the material was allowed to cool, placed in the refrigerator overnight, and then centrifuged to throw down a cohesive, yellowish brown sediment. Up to this point, the procedure was the same as that described by Krasov. However, at this stage the supernatant fluid was separated, and instead of the precipitates being removed at the various pH levels as advocated by Krasov, the fluid was at once adjusted to pH 7.0 with 10N NaOH, whereupon a heavy precipitate formed. This was removed by centrifuging, leaving a clear supernatant fluid. The supernatant fluid was separated and was used as skin-test material. One-tenth volume of 8.5 per cent NaCl and of 5 per cent phenol were added to make the fluid isotonic and to

TABLE 1—Results of Agglutination Tests and Allergic Skin Tests with Hydrolyzate and Sonic Filtrate of Brucella Abortus on Herds Enrolled under Plan B for the Eradication of Brucellosis

Aggl. titers	No. animals	No. animals having allergic skin reactions					
		To BH			To SF		
		P.	S.	N.	P.	S.	N.
P.	190	54	32	104	61	25	104
S.	68	19	13	36	20	11	37
N.	177	26	18	133	33	10	134

P. = Positive. S. = Suspicious. N. = Negative.
BH = Brucella hydrolyzate. SF = Sonic filtrate.

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obtain a final concentration of 0.5 per cent phenol as a preservative. A nitrogen determination was made on the fluid, and 0.85 per cent NaCl containing 0.5 per cent phenol was used as diluent for further dilutions to the desired concentrations of protein. Since this material was obtained by hydrolyzing Brucella cells, this skin-test substance will be referred to as Brucella hydrolyzate (BH). For comparative purposes, SF was used which was prepared according to a method previously described.[5]

EXPERIMENTS

Serologic Tests with Brucella Hydrolyzate.—In the process of preparing the nonagglutinogenic skin-test substance, a portion was removed just before autoclaving, *i. e.*, after washing in alcohol, drying with ether, and resuspending in 0.2N H_2SO_4. When adjusted to pH 7.0 and diluted with 0.85 per cent NaCl to correspond to the density of standard agglutinating fluid, this suspension of Brucella cells was satisfactorily agglutinated by Brucella antiserum, and not by a known negative serum. Furthermore, this material also bound complement in a complement-fixation test with a positive Brucella serum, but not with a negative serum. On the other hand, material from the same batch after being autoclaved and adjusted to pH 7.0, either with the precipitate suspended or after removal of the precipitate, did not bind complement at all when tested against a positive serum. It would appear, therefore, that autoclaving *Br. abortus* at a low pH breaks down the organisms, and the part which goes into suspension not only loses its antigenic property, but its ability to bind complement as well.

Studies of Antigenicity of Brucella Hydrolyzate in Rabbits.—In order to determine the antigenic property of the product before and after autoclaving, two groups of 6 rabbits each were injected respectively with the Brucella suspension after being washed with alcohol and ether and suspended in 0.2N H_2SO_4, just before autoclaving, and the clear supernatant fluid obtained after autoclaving by adjusting the material to pH 7.0 and removal of the precipitated hydrolyzate. The two products in 0.3-cc. quantities containing 1 mg. of protein were injected intracutaneously into the two respective groups of rabbits at weekly intervals for five successive weeks. Each weekly dose was injected in the form of 0.1 cc. into three sites at the same time, and blood for agglutination tests was obtained from the animals before each intracutaneous injection. Injections of the nonautoclaved material stimulated the production of agglutination titers ranging up to 1:640, while the rabbits injected with the hydrolyzate were all negative to the agglutination test in 1:20 dilution of their serums, the lowest dilution used throughout the experiment. In addition, the injections of Brucella suspensions caused the production of an edema with induration which persisted for many days while the intracutaneous injections of BH did not produce visible reactions when the animals were examined daily for several days. Two and one-half months after the last weekly injection, the rabbits which had been injected with BH were reinjected with one-third the original dose, 0.1 cc. containing 0.33 mg. of protein, of the homologous substance to determine whether the previous injections had sensitized them to subsequent skin tests, and no reactions were observed. Thus, large doses of BH when injected intracutaneously into rabbits did not stimulate the production of agglutinins and did not sensitize the animals to a subsequent skin test.

Studies of Antigenicity of Brucella Hydrolyzate in Cattle.—After the experiment on rabbits, the antigenicity of BH was studied in cattle. Eighteen cows negative to the agglutination test for brucellosis were given repeated intracutaneous injections of BH at monthly and bimonthly intervals, the total quantity of protein per injection ranging from 1 to 12 mg. Ten of the animals received as many as eight injections. During this study blood samples were obtained for agglutination tests at weekly intervals, and the serums were consistently negative in a 1:25 dilution when tested against Brucella antigen. Furthermore, no allergic skin reactions were noticed at the sites of injection when examined at 24, 48, and 72 hours, which would indicate that, as in the findings in rabbits, the intracutaneous injections of the material did not sensitize these animals to subsequent skin tests.

Intracutaneous Tests for the Detection of Brucellosis in Herds with Brucellosis of Long Standing.—After BH had been found nonantigenic, its ability to evoke allergic skin reactions in cattle with brucellosis was investigated. In order to be able to compare the results of the intracutaneous test with hydrolyzate with those obtained with SF, seven herds enrolled under plan B for the eradication of brucellosis were studied. It was necessary to limit this comparative study to such herds because in these animals the production of agglutinins as a result of injections of SF was not particularly objectionable, *i.e.*, cattle enrolled under plan B are not slaughtered when found positive to the agglutination test, and no indemnity is paid for them. Four hundred and thirty-five tests were carried out with BH and SF. The technique used was the same as that employed in the intradermal tuberculin test. The BH was injected into the skin of the tail fold and that of the vulva on one side, and the SF was injected simultaneously into the skin of the tail fold and that of the vulva on the other side. The quantity

of BH injected into each site was 0.2 cc. containing 0.5 mg. of protein, and that of SF was 0.2 cc. containing 0.2 mg. of protein. This concentration of SF had previously been found as satisfactory as larger doses, and in order not to produce too high agglutination titers as a result of intracutaneous injections of the material into previously negative animals, this dose was adhered to in the tests. In the case of BH, larger quantities could be used because the substance was nonantigenic, but 0.2-cc. doses containing 0.5 mg. of protein were decided upon since in comparative tests with varying concentrations of the material no advantage was found in using larger doses. Positive reactions with either of the two skin-test substances manifested themselves by the presence of edema with induration at the sites of injection, and they reached their height in forty-eight to seventy-two hours. Only when the injected tail fold increased to twice its normal thickness or more, or the swelling in the vulva reached the size of a pea or larger dimensions, was the reaction considered positive. Smaller swellings were considered suspicious reactions. At the time of the skin testing, blood samples were obtained from the animals for the purpose of determining their agglutination titers, and milk samples were collected and cultured for the presence of *Br. abortus*.

The results of the agglutination tests and the allergic skin tests with BH and SF are shown in table 1.

These data show in general good correlation between the results obtained with BH and SF. The main discrepancy appears when the findings of the agglutination tests on one hand are compared with the results of the intracutaneous tests on the other, since more than half of the groups positive or suspicious to the serologic test were negative to the intracutaneous test with both materials. Furthermore, it was noted that the majority of the animals positive to both the serologic and the allergic tests had agglutination titers of 1:100 or 1:200, while more than 50 per cent of those positive to the agglutination test and negative or suspicious to the skin test had titers ranging from 1:400 and up as shown in table 2. In this latter group, *Br. abortus* was isolated from the milk in 17 instances. These findings would suggest that the more severe the infection, as judged by the agglutination test and milk cultures, the less likely is there to be a demonstrable skin sensitivity, perhaps due to desensitization. It is known that in cattle with extensive tuberculosis the tuberculin test may be negative, and Topley and Wilson[6] in discussing human tuberculosis state that patients in an advanced stage of tuberculosis generally give a negative tuberculin reaction. As shown in table 1, about 25 per cent of the animals negative to the agglutination test yielded positive or suspicious skin reactions. Such findings were also obtained in previous studies with SF[1, 2] on herds with brucellosis of long standing, and they may be due to past infection which the animals had overcome to an extent that they lost their agglutination titers partially or completely, or to a latent or transient infection which produced an allergic skin sensitivity. According to Huddleson,[7] an allergic skin sensitivity may persist in people after they have overcome a Brucella infection. Further evidence to this effect was obtained by intracutaneous tests on animals which had been vaccinated with strain 19 vaccine, and which will be discussed below. The presence of Brucella infection in animals with negative agglutination titers and positive skin reactions could not be demonstrated by milk cultures in the cases examined.

Intracutaneous Tests on Animals Vaccinated with Strain 19 of Brucella Abortus.—Animals in the above herds which had been vaccinated as calves were also subjected to the intracutaneous test with BH and SF. The time interval between vaccination and testing of these animals varied from three weeks to twenty-two months. In general, the heifers which were tested soon after vaccination had positive blood serum titers while the skin reactions were negative or suspicious in the majority of cases. In those animals in which the interval

TABLE 2—Comparison of Agglutination Titers and of Results of Milk Cultures for Brucella Abortus, with the Degree of Allergic Skin Reactions to Brucella Hydrolyzate

	No. animals	No. animals with Brucella-positive milk cultures*	% of animals having indicated agglutination titers					
			1:100	1:200	1:400	1:800	1:1,600	1:3,200
Positive aggl. titer..... Positive skin reaction.....	54	5	37.0	35.2	18.5	5.6	3.7	..
Positive aggl. titer..... Suspicious skin reaction.....	32	6	9.4	31.3	21.9	25.0	12.5	..
Positive aggl. titer..... Negative skin reaction.....	104	17	10.6	34.6	15.4	25.0	10.6	3.8

*Number of animals from whose milk *Br. abortus* was isolated at the time of the blood and skin testing.

between vaccination and testing was longer, the agglutination titers were comparatively lower, and the proportion of animals with positive skin reactions increased. Finally, those animals which were furthest removed from the time of vaccination were negative to the agglutination test but, in some instances, still retained an allergic skin sensitivity. These findings are in accord with the observations of Evans *et al.*[8] that in human brucellosis the allergic state usually develops later than detectable agglutinins, and is generally retained for longer periods after recovery. Table 3 contains the results of testing of 139 vaccinated animals.

TABLE 3—Results of Agglutination Tests and Allergic Skin Tests with Hydrolyzate and Sonic Filtrate of Brucella Abortus on Animals which Had Been Vaccinated with Strain 19 during Calfhood

Aggl. titers	No. animals	No. animals having allergic skin reactions					
		To BH			To SF		
		P.	S.	N.	P.	S.	N.
P.	16	5	4	7	5	3	8
S.	28	13	4	11	13	3	12
N.	95	17	11	67	18	7	70

P. = Positive. S. = Suspicious. N. = Negative. BH = Brucella hydrolyzate. SF = Sonic filtrate.

Intracutaneous Test on Cattle under Plan A for the Eradication of Brucellosis. — When testing animals in herds enrolled under the test-and-slaughter method for the eradication of brucellosis, SF could not be used as skin-test material for reasons previously discussed. Therefore, only the BH was injected intracutaneously into the skin of a tail fold and that of the vulva in 0.2-cc. quantities containing 0.5 mg. of protein. In such herds official agglutination tests are carried out at comparatively frequent intervals, and any animal found positive is eliminated. Thus, the cattle found positive to the agglutination test in this study may be assumed to have become infected recently because they had been tested only three months after a previous test. Table 4 shows the results on 161 animals in two herds. Of these 7 were positive, 7 suspicious, and 147 were negative to the agglutination test. All the animals negative to the blood test were also negative to the allergic skin test, while the 14 cattle with positive and suspicious serologic titers yielded negative skin reactions in 9 cases.

DISCUSSION

Various attempts have been made in the past to determine the practicability of an allergic skin test for the detection of brucellosis in the bovine species. Hutyra, Marek, and Manninger[9] state that in practice the allergic test has not so far been generally adopted. In addition to the fact that the products which had been used for this purpose, *i.e.*, killed suspensions of Brucella, extracts of the organisms, and filtrates of broth cultures, failed to diagnose all cases of brucellosis, they also stimulated the production of agglutinins in Brucella-negative animals. In previous studies[1,2] with sonic filtrate of *Br. abortus* (SF) as a skin-test material, the same objectionable features were encountered, and it was soon realized that to be of any possible value, the allergic skin test would have to be applied in conjunction with the agglutination test. The antigenic property of SF, however, prevented the investigation of such possibility until a nonagglutinogenic product from *Br. abortus* could be prepared; namely, by modifying the method described by Krasov,[4] according to which cells are suspended in 0.2N sulfuric acid and autoclaved, a Brucella hydrolyzate (BH) was obtained which was nonagglutinogenic, but which was very similar to SF in producing allergic skin reactions. However, the allergic tests with both products yielded negative reactions in 54.7 per cent of 190 cattle found positive to the agglutination test in herds with brucellosis of long standing. On the other hand, of 177 animals negative to the agglutination test, 44 cows, or 24.8 per cent, responded with a positive or suspicious reaction to the intracutaneous test with BH, and a similar number showed such reactions to SF. That these reactions with BH cannot be considered nonspecific was shown by studies on control cows which were known to have had no contact with brucellosis. Eighteen such animals were tested repeatedly, some of them as frequently as eight times at one- and two-month intervals, with negative results. Fur-

TABLE 4—Results of Agglutination Tests and Allergic Skin Tests with Brucella Hydrolyzate on Herds Enrolled under Plan A for the Eradication of Brucellosis

Aggl. titers	No. animals	No. animals having allergic skin reactions to hydrolyzate		
		P.	S.	N.
P.	7	1	3	3
S.	7	1	..	6
N.	147	..	..	147

P. = Positive. S. = Suspicious. N. = Negative.

thermore, when animals were tested in herds in which frequent agglutination tests were carried out, and the positive cattle were eliminated, all the cows negative to the agglutination tests were likewise negative to the allergic skin test. Thus, one is led to the assumption that the animals with negative agglutination titers and positive skin reactions in the herds mentioned above had either overcome an infection, but retained the skin sensitivity, or that the allergic state was due to a latent or transient infection. In support of the former view, it was found in a study on vaccinated animals that some animals vaccinated as calves with strain 19 still showed an allergic skin sensitivity long after vaccination, at a time when no demonstrable agglutinins were present in the blood serum. These data would suggest that it is possible for an animal to lose the agglutination titer while retaining an allergic skin sensitivity. Whether it is possible for an animal to become sensitized due to a latent or transient infection, or merely by coming in contact with infected material, is an open question. Meyer and Geiger[10] found that the percentage of positive skin reactions in people was correlated with the frequency of contact with infected animals, and Heathman[11] determined by investigating the skin sensitivity in packing house employees that the incidence of the allergic state increased with the length of service without the presence of symptoms of the disease. Although no exhaustive study was made, *Br. abortus* was not at the time of testing isolated from the milk of the cows negative to the agglutination test and positive to the skin test.

It is apparent from the above results that the skin test alone cannot be depended on to diagnose brucellosis in cows, because more than half of the animals with positive agglutination titers did not show an allergic skin sensitivity. The advisability of the simultaneous use of both the agglutination and the allergic test would only be warranted if, by this combination, more infected animals would be detected than by the serologic test alone. With the use of the nonagglutinogenic BH, entire herds of cattle could be tested, but in this study no advantage was found in the use of both tests combined because in herds in which the blood test is carried out frequently, and the reactors are eliminated, all the animals negative to the agglutination test were also negative to the skin test. In herds with permanent brucellosis, on the other hand, of 44 cows with negative agglutination titers and positive or suspicious allergic reactions, not one animal could be proved infected by isolation of *Br. abortus* from the milk. It is of interest to note that Edwards and Hull[12] reported similar findings in their studies of an intradermal test with pullorin for the detection of pullorum disease; namely, 52.4 per cent of the birds which were positive to the agglutination test were negative to the intradermal test, while 54.3 per cent of those that reacted to the intradermal test were negative to the serologic test. Furthermore, they found at autopsy that a very large percentage of the birds with positive intradermal reactions and negative agglutination titers were not infected with *S. pullorum.* Beach and Michael[13] in a similar study demonstrated that 7.1 to 55.5 per cent of birds infected with *S. pullorum* failed to react to the pullorin test and that they obtained positive pullorin tests in 13.8 to 43.1 per cent of noninfected birds.

SUMMARY

A nonagglutinogenic hydrolyzate prepared from *Brucella abortus* (BH) was studied from the standpoint of detecting allergic skin sensitivity in cattle. When used on herds with brucellosis of long standing, of 190 animals with positive agglutination titers, 54.7 per cent were negative to the intracutaneous injection of BH, while of 177 cows negative to the agglutination test, 24.8 per cent yielded positive or suspicious skin reactions. The results obtained with BH correlated closely with those observed with the use of sonic filtrate of *Br. abortus* (SF) on the same animals.

When the intracutaneous test with BH was applied to herds under the test-and-slaughter method for the eradication of brucellosis, all the cattle negative to the agglutination test were devoid of allergic

skin sensitivity, while of 14 animals with positive or suspicious serologic titers, 9 were negative to the skin test.

Animals vaccinated during calfhood with strain 19 appeared to retain an allergic skin sensitivity in a considerable percentage of cases at a time when their blood serum titers had returned to a negative status.

That the reactions produced by the intracutaneous injections of BH were specific was demonstrated by the fact that not one animal with a negative history and negative to the agglutination test was positive to the skin test. However, because of the discrepancies between the results of the serologic and the intracutaneous tests, the latter cannot be relied upon to diagnose individual cases of brucellosis. The allergic skin test with the nonagglutinogenic BH might be of value in surveys to detect the animals that have been in contact with infected sources.

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Independent Variation of Characteristics in Brucella Abortus Variants and Their Detection

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DETAILED descriptions of a number of variants of *Brucella abortus* have been presented by Henry,[1] Plastridge and McAlpine,[2] Morales-Otero,[3] and Huddleson.[4,5] The work of these authors has been of great value in the recognition of the dissociative aspects of the genus Brucella, particularly in relation to the problems of immunization. However, some of their ideas regarding the existence of successive orderly changes ("cyclogenic development," "course of dissociation," "cycles," "successive phases") appear to require revisions in view of recent information on the rôle of inherent factors and selection in bacterial variation.[6,7,8] It now appears that dissociation is caused by the spontaneous appearance of a small number of variants (mutants) which can establish themselves if they have a higher selection value than the original members of the population. The types of variants which can arise seem to differ between clones (inherent differences), and the selection value of the arising variants is greatly modified by environmental conditions. These factors alone can contribute to the occurrence of *apparently* successive orderly changes (for further details see Braun[7,8]). In contrast to cyclogenic interpretations of dissociation, the interpretation of bacterial variation in terms of spontaneous mutations requires the existence of possible independent changes in a set of characteristics which are not based on one common causative factor, *e.g.*, colony appearance and physiologic behavior. Independent changes of this nature have been described to some extent for *Br. abortus* (especially by Huddleson[5]) and in considerable detail for some other species, *e.g.*, *Klebsiella pneumoniae*.[9] Additional information on the detection of independent variation of some characteristics in *Br. abortus* will be reported here, and the significance of this mode of variation in the work on control of brucellosis will be pointed out.

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From the Department of Veterinary Science, University of California, Berkeley.

A. THE ACRIFLAVINE TEST*

In order to detect independent changes in characteristics it is necessary to supplement morphologic observations by a test which indicates nonmorphologic changes. The acriflavine† test has proved to be of great value for this purpose. This test was first applied to studies with Brucella by Alessandrini and Sabatucci [10, 11] but has not been as widely used in Brucella studies as in work on Salmonella. [12, 13, 14]

Neutral acriflavine was made up for use in a 1:1,000 aqueous solution and stored in brown bottles. It was used in either of two ways, by suspending in saline the growth from a slant and testing the suspension macroscopically after addition of acriflavine solution, or by picking a single colony and observing the results microscopically on a slide after addition of acriflavine solution. Both methods can be carried out easily and rapidly, the microscopic method being preferred for routine work. In the macroscopic or tube test, the growth is washed (not scraped, *cf.* Hirsch,[13] on the effect of colloidal agar upon the acriflavine test) from the slant and suspended in physiologic saline. Then 1.0 cc. of this suspension is placed in a small agglutination tube and 0.1 cc. of the 1:1,000 aqueous solution of acriflavine is added. If the culture is antigenically smooth (S) no change occurs; the suspension remains uniformly turbid. If, however, the culture is antigenically rough (R) a flocculent precipitate will appear almost immediately, and if the tube is allowed to stand at room temperature for an hour or two this precipitate will settle out, leaving the supernatant fluid completely clear. In the case

*The studies with acriflavine were carried out by A. E. Bonestell.

†Acriflavine is also known as trypaflavine. It is an alkaloid compound derived from coal-tar and is a mixture of 2.8-diamino-10-methylacridinium chloride and 2.8 diaminoacridine. The compound used in the studies here reported has been Neutral Acriflavine, U.S.P., made by the Abbott Laboratories.

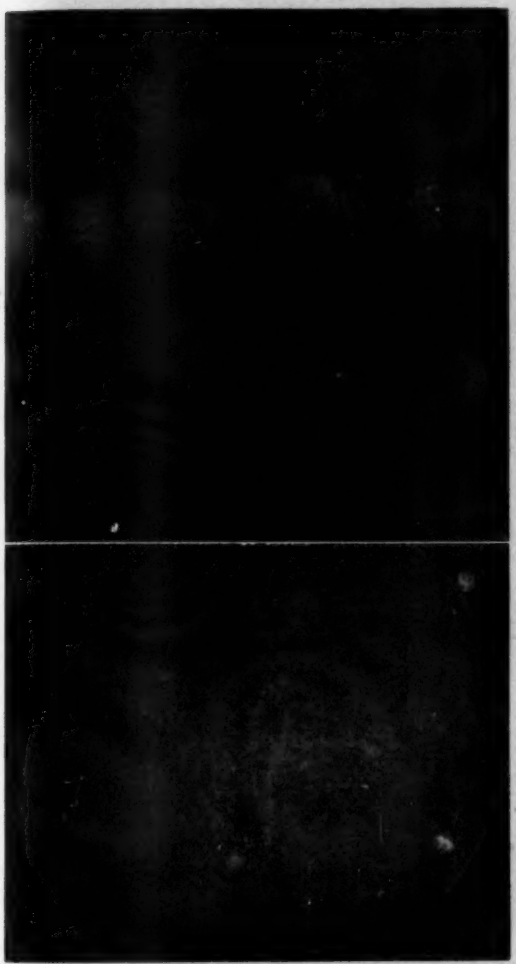

Fig. 1A—S colonies and the result of an acriflavine test made with one of these S colonies. x20.

of mucoid (M) cultures (which frequently have been termed brown (Br), in *Br. abortus*), a slimy precipitate is formed, while the so-called intermediate (I) variants either form no precipitate or show a flocculation consisting of particles which are smaller and more granular than those obtained with R cultures. In the more rapid microscopic test, a single colony is picked from a plate and emulsified on a slide in a drop of physiologic saline. Care should be taken to choose the material to be tested from areas which show well-isolated colonies and to use plates which are not older than five days. It has been observed that the presence of dead cells will modify the results of the acriflavine test and an error can, therefore, be introduced if material is tested from areas with crowded growth or from plates which have been allowed to age. To the emulsified material, a drop of the acriflavine solution is then added and thoroughly mixed with a

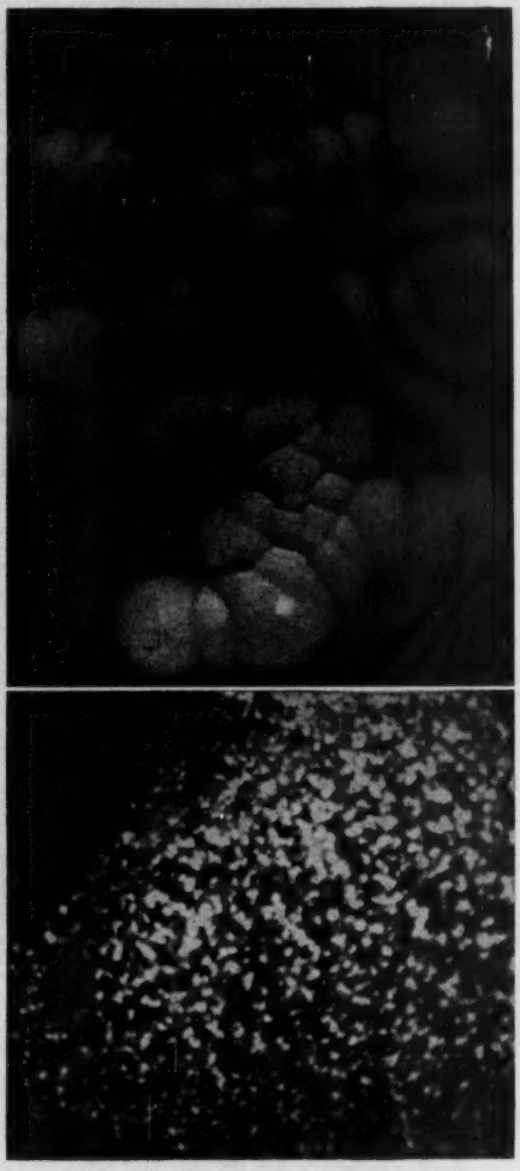

Fig. 1B—R colonies and the result of an acriflavine test made with one of these R colonies. x20.

loop or applicator stick. If the colony is antigenically smooth the drop remains homogeneous throughout. Examination with a dissecting microscope will reveal Brownian movement, which persists if the slide is sealed to prevent evaporation. If the colony is antigenically rough, clumping and flocculation will result after addition of the acriflavine solution and all Brownian movement will cease. In the case of antigenically mucoid colonies, the reaction will resemble somewhat the previously described rough reaction, but can be distinguished by the appearance of a more

slimy flocculation, which often forms thread-like configurations. These different reactions are illustrated in figures 1A to 1E.

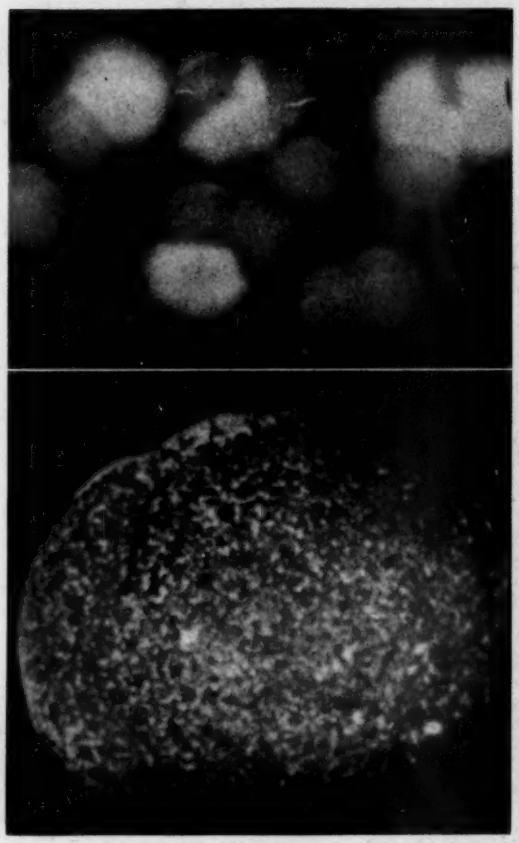

Fig. 1C—S^R and R colonies and the results of an acriflavine test made with one of the S^R colonies. x20.

B. THE VARIANTS

A number of variants of *Br. abortus* which will establish themselves under standard *in vitro* conditions have been analyzed with the help of the acriflavine test. A detailed description of these variants appears unnecessary since most have been well described by Henry[1] or Huddleson.[4, 5] In the following section emphasis will be placed primarily on the presence or absence of linkage between colony appearance and the results of acriflavine tests.

1) *The True S Types—(fig. 1A).*—Colonies appear smooth. The acriflavine test gives the typical smooth reaction. This type (strain 19S) is commonly employed in the manufacture of vaccine. It can arise in certain R populations.

2) *The Intermediate Types.* ‡—These variants establish themselves in certain smooth and rough populations. They appear to comprise a group of mutants which range from close resemblance to S to close resemblance to R. They are usually quite unstable, i.e., they are rapidly suppressed by arising S or R mutants. Occasionally, however, I populations are observed which appear to have a considerably higher selection value than either S or R populations and thus remain predominantly I. The acriflavine test gives varying results (see above).

‡The term intermediate was originated by workers who believed in a cyclogenic interpretation of dissociation and interpreted these types as intermediate stages in the transition from S → R.

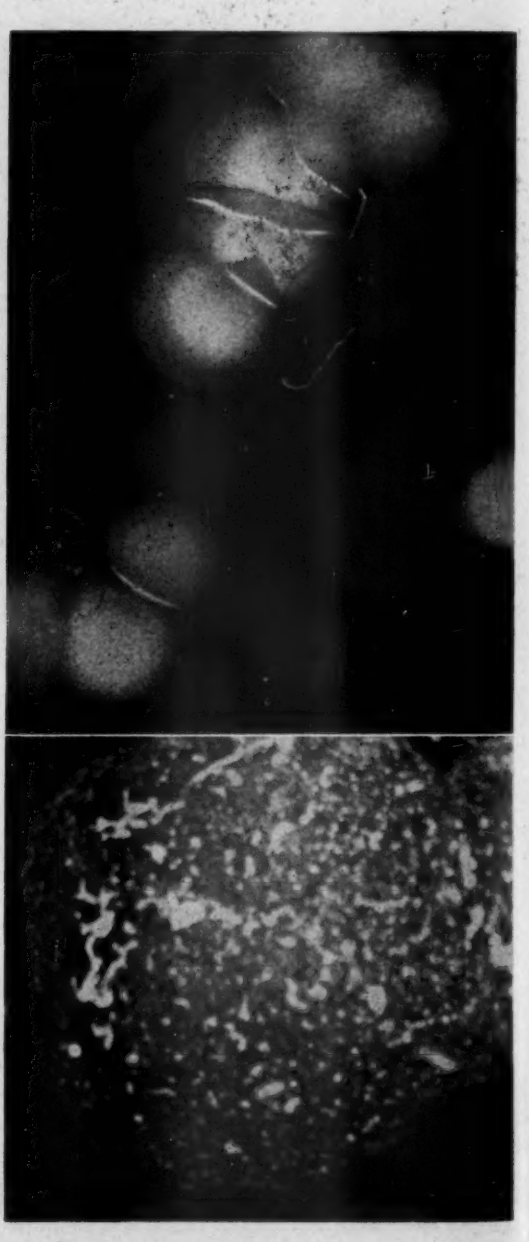

Fig. 1D—M and S colonies and the result of an acriflavine test made with one of the M colonies. x20.

3) *The R Types (fig. 1B)*—Colonies appear rough. The acriflavine test gives the typical rough reaction. Rough mutants usually have a higher viability *in vitro* than S types and thus can establish themselves in S populations.[6,7] There appear to arise quite a number of R types differing in degree of colony roughness.

3a) *The S^R Type (fig. 1C)*—This variant has been repeatedly observed to arise in R populations. Some evidence points to its occasional appearance in S populations. The colonies are indistinguishable from S colonies. The acriflavine test gives a typical rough reaction. S^R mutants have a higher selection value than R types.

4) *The Mucoid Types (fig. 1D)*.—These variants have been observed to arise in S and in R populations. The colonies of the most frequently encountered M type (probably identical with Huddleson's Ma2) have a brownish center and a transparent outer sphere. Old colonies of this type lose the transparent sphere and attain a more uniform brownish, rough appearance. Another M type (probably identical with Huddleson's Ma3) is almost uniformly transparent. Other M types which differ in prominence of their transparent sphere and degree of coloring have been observed. All these types give a typical mucoid reaction with the acriflavine test.

4a) *The S^M Type (fig. 1E)*.—This variant has been isolated from M populations. The colonies are difficult to distinguish from S colonies. The acriflavine test gives a typical mucoid reaction.

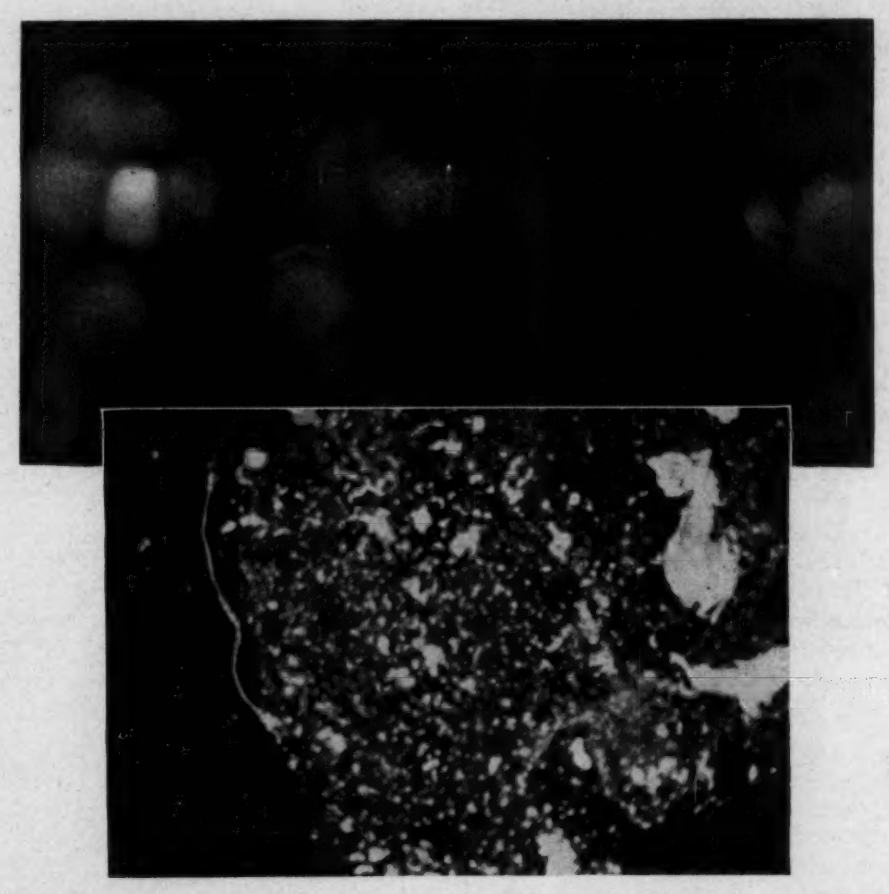

Fig. 1 E—S^M and R colonies and the result of an acriflavine test made with one of the S I colonies. x 20.

DISCUSSION

The occurrence of variants which differ in colony morphology but show identical acriflavine reactions (*e.g.*, R, S^R, or M, S^M) amply demonstrates the existence of inde-

§In recent animal experiments, Dr. S. Hauge has confirmed that the results of the acriflavine test parallel the antigenic activity of the variants *in vivo*.

pendent variation of characteristics during dissociation in *Br. abortus*.§ This evidence furnishes additional support for the interpretation of dissociation in terms of spontaneous, undirected appearance of variants (mutation) and their subsequent establishment under selection pressure. In the present report only one pair of characteristics has been analyzed, namely, colony appearance and results of the acriflavine test, but similar analysis for additional characteristics would probably reveal the existence of many other possible independent changes. (For a discussion of possible causative mechanisms of independent variation see Braun[8]). It is realized that in most instances a change in colony morphology is linked with antigenic changes, but the above described S^R and S^M types exemplify that this is not always the case. This fact makes it unsafe to rely on morphologic observations alone for classification of variants, a point which is of special significance in vaccine production. The true S, S^R, and the S^M types all look alike when their colonies are viewed under the microscope, but differ significantly in their antigenic composition. Therefore, as long as the true S type of *Br. abortus* alone is recognized as valuable for immunizing purposes, it is advisable that morphologic observations be supplemented by tests, such as the acriflavine test, during the manufacture of vaccines.

SUMMARY

The occurrence of independent variation of characteristics during dissociation of *Brucella abortus* has been exemplified by observations on changes in colony morphology and changes in antigenic behavior as revealed by the acriflavine test. The observations support the interpretation of dissociation in terms of spontaneous appearance (mutation) of variants and their establishment under selection pressure. It is pointed out that, in the presence of independent morphologic and other changes, morphologic observation alone cannot be relied upon for correct classification of variants.

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A Note on the Comparison of Gases from Spontaneous and Rumen Fermentation of Certain Feedstuffs

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ONE of the phases of digestion in ruminants which has not received adequate attention by research workers is that of gas production, absorption, and elimination. The gases of digestion cause astounding losses annually. Livestock raisers know well the serious consequences of acute tympany or so-called bloat. The exact cause of bloat has not been determined.

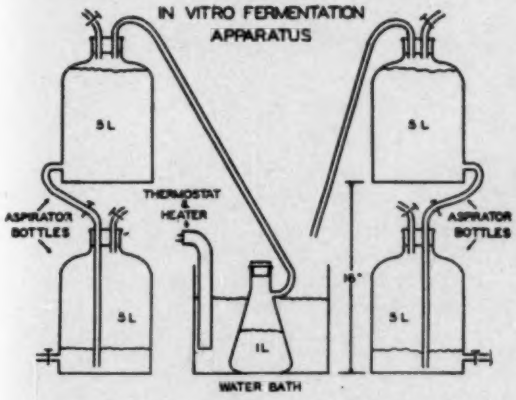

Fig. 1—In vitro fermentation apparatus.

Divers theories have been published in the literature but all of them seem to lack convincing evidence necessary to defend them. One does not look upon disturbed physiology with the same singularity as a disease caused by an infectious agent. The conditions leading to bloat are multiple and complex, yet when they become known they may seem simple. It seems logical that learning more of the basic physiology of gas in digestion should bring about better understanding of the complex processes which may be accepted as the causes of bloat. It was reasoned that an approach to the study of gas production, assimilation, and elimination in ruminants should include a study of the gases elaborated by feedstuffs fermented naturally *in vitro*. It is obvious that the bacteria responsible for the fermentation enter the rumen with the feed and are an important part of the bacterial content of the rumen. Many reports have been published about the kinds and relative percentages of gases in the rumen. A review of this literature is well reported by Cole, *et al.*[1]

Experimental Methods in Vitro

The apparatus assembled to collect the gases produced from spontaneously fermenting feedstuffs is shown in figure 1. One hundred grams of feedstuff was placed in the 1-liter suction flask and diluted with 300 to 500 cc. of distilled water. The flask was placed in a water bath at body temperature and the side arm of the flask was connected by rubber tubing to the upper aspirator bottle. Allowing the confining fluid to pass from the upper aspirator bottle to the bottom one created enough suction to draw the gas as produced into the upper bottle where it could be measured at intervals.

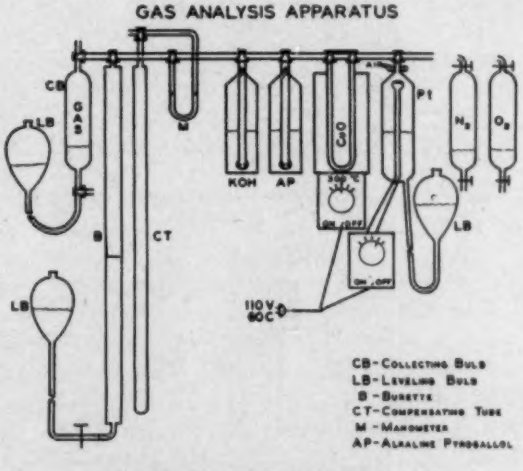

Fig. 2—Gas analysis apparatus.

Aliquots of the gases produced were taken at intervals and analyzed for their chief constituents. The apparatus used was a Fisher Precision Mine Gas Analyzer of the Orsat

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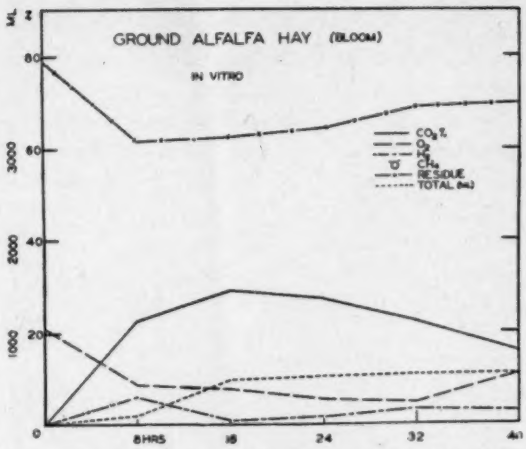

Fig. 3—In vitro fermentation gases of alfalfa hay (1945 bloom).

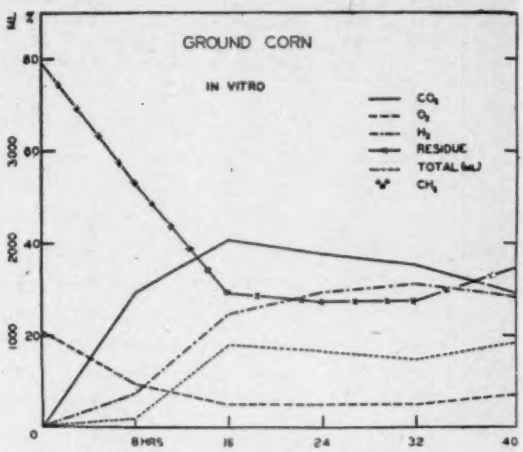

Fig. 4—In vitro fermentation gases of ground corn.

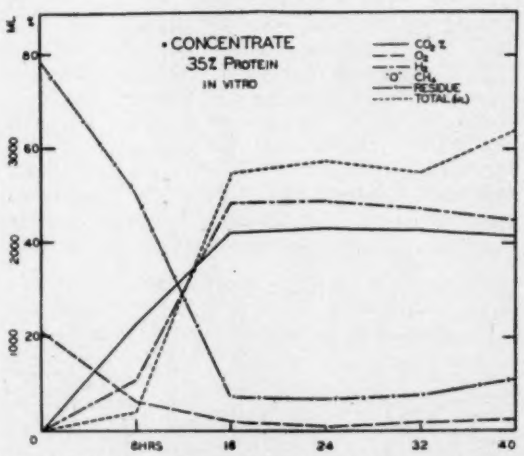

Fig. 5—In vitro fermentation gases of concentrate (35% protein).

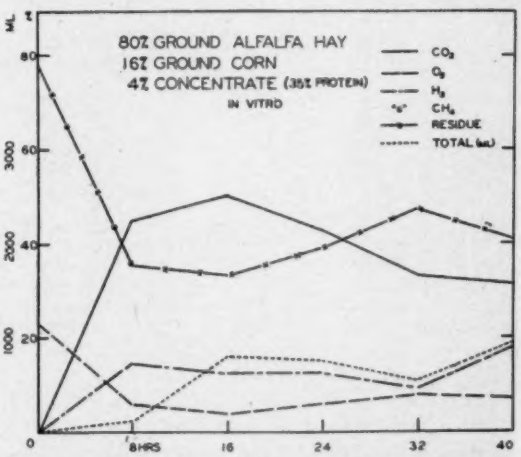

Fig. 6—In vitro fermentation gases of a mixture of 1945 bloom alfalfa hay, 35% protein concentrate, and ground corn.

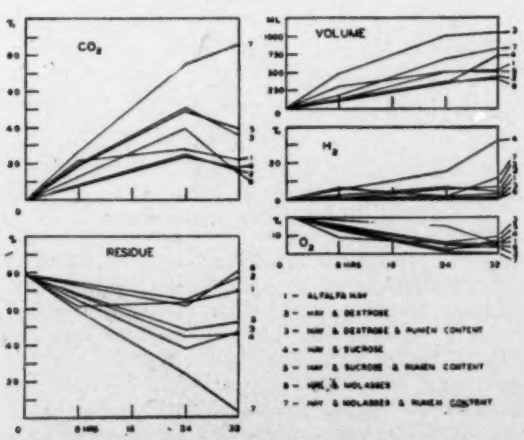

Fig. 7—In vitro fermentation gases of 1945 bloom alfalfa hay and carbohydrates.

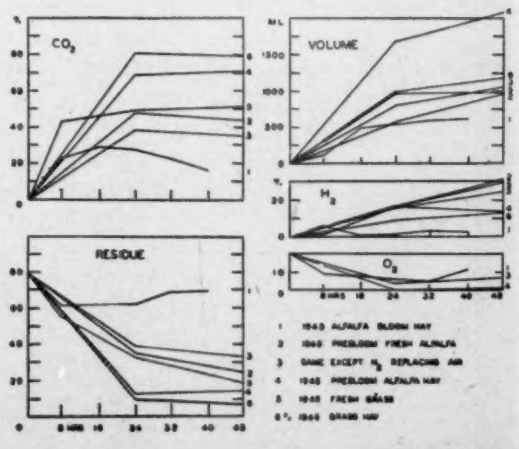

Fig. 8—In vitro fermentation gases of 1945 bloom alfalfa hay, 1946 prebloom fresh alfalfa, 1946 fresh young grass, and hays from the fresh products.

type shown schematically in figure 2. Fifty per cent potassium hydroxide was used to remove carbon dioxide; alkaline pyrogallol was employed to remove oxygen. Hydrogen was oxidized over hot copper oxide. Methane, determined by burning over a hot platinum wire, was not found in *in vitro* samples. Carbon monoxide, present in very small amounts, was extracted during hydrogen oxidation. The residue was the gas that remained after removal of the above named gases. The feedstuffs used included:

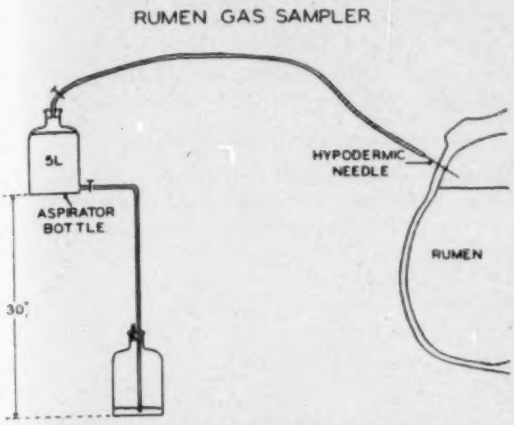

Fig. 9—Rumen gas sampling equipment.

1945 second-cutting bloom alfalfa hay.
Ground corn.
A 35 per cent protein concentrate.
1946 first growth prebloom fresh alfalfa.
Laboratory-dried hay from the above mentioned fresh alfalfa.
1946 fresh young native grass.
Laboratory-dried hay from the above mentioned grass.
Dextrose, sucrose, molasses, or fresh rumen content.

When these sugars or fresh rumen contents were included with the hay fermented *in vitro* these were added in 10-Gm. amounts.

RESULTS IN VITRO

The results of about 1,400 determinations of gases produced by spontaneous fermentation of the feeds mentioned are depicted in graph form in figures 3 to 8. Study of these data reveals some interesting observations:

1) The feedstuffs fermented *in vitro* produced similar gases but in different quantities.

2) During the fermenting process, the atmospheric oxygen over the mediums decreased considerably within the first eight hours and thus rendered the process more anaërobic in character.

3) Hydrogen was produced more slowly than carbon dioxide (fig. 3 to 8).

4) More total gas was produced from the concentrate than from the corn and more from the corn than from the hay (fig. 3 to 5).

5) Carbon dioxide was produced more abundantly in fermenting hay and corn, but hydrogen gas was most abundant in fermenting protein concentrate (fig. 3 to 5).

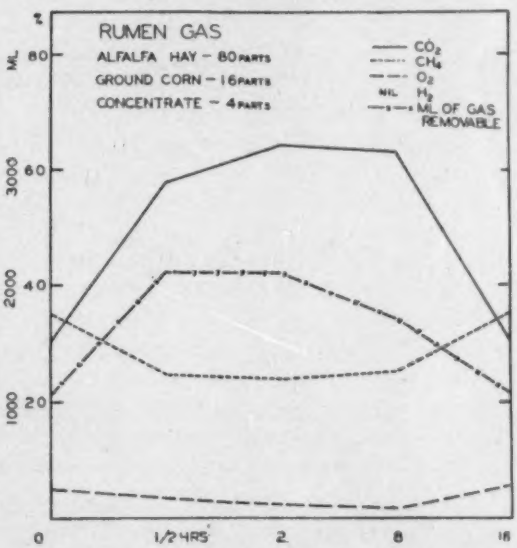

Fig. 10—Rumen gas on hay, corn, and concentrate.

6) A mixture of hay, concentrate, and corn in the proportions fed later yielded results which one might expect considering the relative quantities used. Total gas and proportions of gases generally corresponded to the feed which was used in greatest quantity, the hay. Carbon dioxide, although produced in greater proportion than any of the three components separately, had a lower total than any of the three except for hay (fig. 3 to 6).

7) No methane was produced *in vitro*.

8) Introduction of 10-Gm. quantities of dextrose, sucrose, or molasses alone did not increase the carbon dioxide in fermenting hay, but addition of the carbohydrates plus rumen content did (fig. 7).

9) Volumes of gas from hay, dextrose, rumen content; from hay, molasses, and

rumen content; and from hay and sucrose were higher than from hay alone while the others were lower (fig. 7).

10) Hay and sucrose produced a relatively high percentage of hydrogen but still considerably less than concentrate alone (fig. 7).

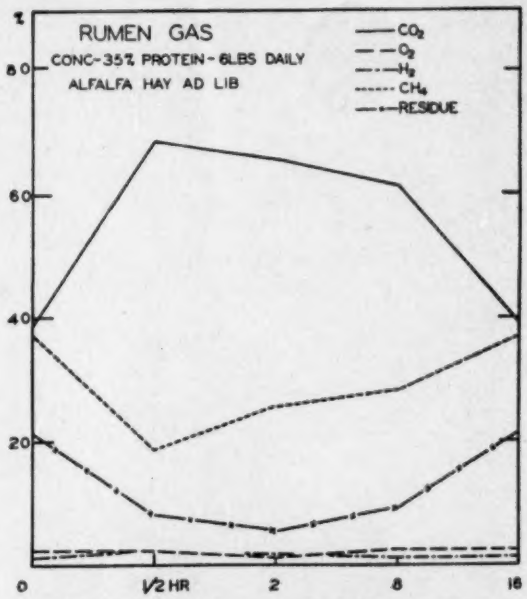

Fig. 11—Rumen gas on hay and concentrate.

11) Compared to 1945 second-cutting bloom hay, 1946 first growth pre-bloom fresh alfalfa, the same fermented under nitrogen instead of air, hay from the above mentioned fresh alfalfa, 1946 young fresh grass, and hay from the fresh grass, all yielded more total gas, more carbon dioxide, and more hydrogen (fig. 8).

12) The hays from the fresh alfalfa and fresh grass were higher in carbon dioxide percentage and lower in hydrogen percentage than the fresh products (fig. 8).

13) The highest relative hydrogen producers were the fresh alfalfa and fresh grass, more so than the hays from these fresh products (fig. 8).

14) Those feedstuffs which usually contained a relatively high protein content, *i.e.*, the concentrate, young fresh alfalfa, and young fresh grass, were relatively high hydrogen producers when fermented spontaneously *in vitro*. Allowing for the fact that 100 Gm. of fresh material contains more water and less actual solid material than the hays from the same material, this result seems even more outstanding. Even the hays from this fresh young alfalfa and grass were greater producers of hydrogen than the standard bloom hay. Apparently, the drying of the fresh alfalfa and grass lowered their ability to produce hydrogen but not their ability to produce carbon dioxide (fig. 3 to 8).

EXPERIMENTAL METHODS IN VIVO

Some of the same feedstuffs fermented in glass were fed to a cow to observe the effects on the gas content of the rumen. Because of absorption and other means of utilizing and eliminating gases produced in the rumen, a measurement of the quantity and kinds of gases is no measure of the amount and kinds actually produced.

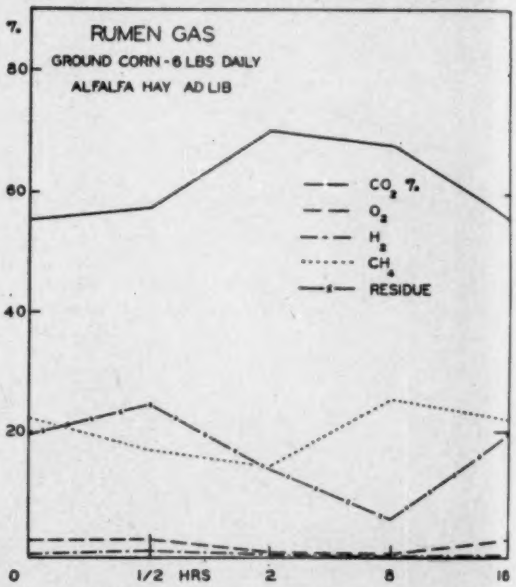

Fig. 12—Rumen gas on hay and corn.

The ration first fed consisted of second-cutting bloom alfalfa hay of good quality, ground corn, and the concentrate containing 35 per cent protein. Hay was kept in front of the cow all of the time, and grain was fed morning and evening. The animal ate about four parts hay to one of grain by weight. The grain mixture was four parts of ground corn and one part of the concentrate, and about 6 lb. were fed. Samples of gases were taken with the apparatus illustrated in figure 9. Only one sample of rumen gas was taken daily. Percentages of components were determined on five samples taken on five different days. Duplicates of all analyses were run.

The animal was then placed on hay and 6 lb. of the concentrate, and the gas sampling and analysis repeated. The same was accomplished on hay and 6 lb. of ground corn. Lastly, the animal was allowed only hay at will. With the feeding of the hay alone, it was necessary to follow the animal's eating, ruminating, and resting periods, and to

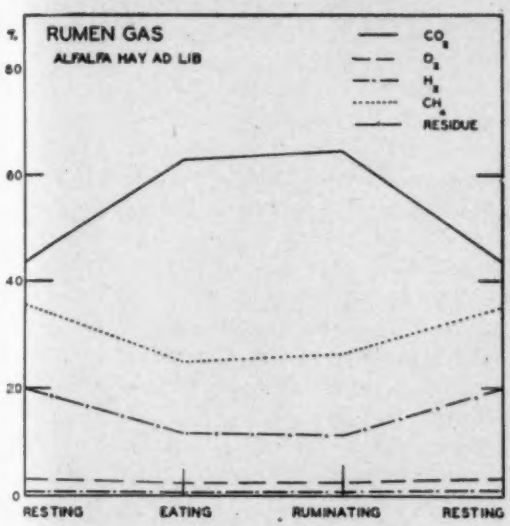

Fig. 13—Rumen gas on hay alone.

sample the gas during these periods. The resting samples were taken in the morning before the cow's first daylight feed. The eating samples were taken about ten to twenty minutes after she started to eat it. The ruminating samples were taken ten to twenty minutes after she started her first daylight rumination. Rations were changed slowly and two weeks were allowed to elapse before sampling was resumed.

RESULTS IN VIVO

Study of figures 10 to 13 reveals that the relative proportions of gases found in the rumen were similar for the different rations except for a slightly lower percentage of methane when the animal was fed hay and corn. The amount of gas capable of being removed from the rumen increased following the feeding of the original mixture. The same increase was observed with the other three rations. It was observed also that the total amount of gas which could be removed when the animal was fed hay alone was less than that of the other three rations.

DISCUSSION

The apparent uniformity of changes in components of rumen gas during a particular feeding régime differed from the comparatively great difference in percentages of components of gas produced from the same feedstuffs by natural fermentation *in vitro*. The two fermenting systems differed. The *in vivo* one was continuous in that new medium, water and organisms were being added, and breakdown products removed constantly. The *in vitro* system was not continuous. Apparently, the breakdown products accumulated limited the process.

The most apparent difference between the kinds of gases produced by fermentation *in vitro* and the kinds of gases elaborated in the rumen was the presence in relatively large quantity of methane in rumen gas and its absence in gases formed in feedstuffs fermented *in vitro* whether inoculated with fresh rumen content or not.

Another striking difference was the low hydrogen percentage in rumen gas as compared to the higher percentages from feedstuffs fermented *in vitro*. These two points are interesting in view of the evidence reviewed by Cole, *et al.*[1] that carbon dioxide and hydrogen may be a source of methane.

The finding of the highest hydrogen percentages in the gases of the higher protein feedstuffs when fermented *in vitro* was interesting considering observations of Deem and his associates[2] that lambs on a high protein grain ration fed sulphur to control overeating may die of hydrogen sulfide bloat. Perhaps, there is a relationship between the hydrogen and sulfur availability and hydrogen sulfide production.

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The Blood Potassium and Calcium Levels of Cattle Grazing Wheat

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THE ECONOMIC problem involved in the loss of cattle grazing winter wheat is of considerable magnitude. Some of the animals which die as a result of grazing such pasture present symptoms which in many respects resemble those of "grass staggers" and "lactation tetany." The symptoms of the former have been adequately described by Sjollema.[1] The condition is characterized by sudden rapid attack, often terminating fatally. The initial symptoms seem to be nervousness and unsteady locomotion with a later violent excitation accompanied by tetanic contractions of the limbs, gnashing of the teeth, rolling of the eyeballs, and frothing at the mouth. The pupils of the eyes are said to be contracted and the animals seem to be blind. Dryerre[2] says, among other things, that the symptoms of "lactation tetany" include an increased respiration rate and temperature elevation of 5 to 6 F. A hyperirritability exists, varying from tremor of the superficial muscles to opisthotonus and extensor spasm. There is champing of the jaws, frothing at the mouth, and retraction of the upper eyelid giving the appearance of exophthalmus. The animals usually die in a few hours. There is an easily recognized similarity in the symptoms described by these two authors.

Any or all animals which die while grazing on wheat are often said to have been suffering from wheat poisoning. The fallacy of such a conclusion is quite obvious. Our observations lead us to believe that the intent of the name "wheat poisoning" applies to those conditions seen in animals grazing wheat which are characterized by hypersensitivity or tetany, or the coma and paralysis resembling parturient paresis. It is these conditions that interest us, and to which we refer in this paper as wheat poisoning. The symptoms are sometimes as follows:

A marked hypersensitivity and tetany exist. The animals are wild, easily excited, and exhibit a desire to attack or repel anyone coming near them. Their ability to do so is hampered by the jerking and incoördination of the legs which are stiff and spread. The eyes appear exophthalmic. The animals run into things, either because they cannot see or because they do not care. The hypersensitivity bears a close resemblance to that seen in strychnine poisoning and tetanus. Some are found down and unable to rise but in a very sensitive, excitable, and frightened attitude. They often attempt to frighten off strangers with sudden movements of the head and by snorting.

On the other hand, most of the cases of "wheat poisoning" which have come to our attention present symptoms more nearly like those of parturient paresis. The animal is in a state of coma or sometimes semicomatose, yet parturition seems in no way to be associated with this condition. Furthermore, these animals have a slight rise in body temperature usually to 102 to 103 F. rather than the subnormal temperature found in parturient paresis. They seem to be in a partial state of starvation, thin, and emaciated as compared with cattle that are doing well. This syndrome is exceedingly difficult to distinguish from that of acetonemia and milk fever.

Hyperexcitability and even tetany are usually characteristic of all these conditions during at least one phase of their development.

Interesting results have arisen from the study of the blood chemistry of grass staggers and lactation tetany. Both conditions are accompanied by low calcium and magnesium contents of the blood of the afflicted animals. The magnesium content is lowered relatively more than the calcium.[1,2]

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McCollum and his coworkers[3] have shown that rats and dogs on diets very low in magnesium show symptoms which are strikingly parallel to those of grass staggers and lactation tetany. There seems to be little doubt that the symptoms of these two conditions are directly due to an altered magnesium content of the systems of afflicted animals. McMillen and Langham[4] maintain that the magnesium content of cattle grazing wheat and suffering from wheat poisoning is not greatly different from that of normal animals.[3]

The ratio of sodium, potassium, and hydrogen ions to calcium, magnesium, and hydroxide ions is known to be important in the maintenance of nervous equilibrium in animals. Potassium is thought to play a special rôle in nerve conduction[5] and is known to be elevated in the blood during certain types of tetany.[6,7] We have accordingly been led to investigate the plasma potassium content of the blood of animals grazing wheat, and to gather additional information about the calcium content of the serum of these same animals.

EXPERIMENTAL PROCEDURE

Samples of blood have been taken in those cases of wheat poisoning which have come to our attention. Since all cases which have been studied were within a short distance of the laboratory, the analyses were started very soon after the samples were collected and conducted immediately to such a point that further standing could have no possible influence on their calcium and potassium contents. Each sample was drawn from the jugular vein into a glass syringe, and immediately divided into two 15-cc. centrifuge tubes. The tubes were filled so that on being stoppered with a rubber stopper they were completely full. Those samples which were to be used for potassium determinations were prevented from clotting by the use of the minimal amount of highly purified sodium oxalate and the potassium determination run on the plasma by the method of Breh and Gaebler[8] supplemented by the diazotization procedure of Briggs.[9] The calcium content was run on serum by the Clark and Collip modification of the Kramer-Tisdall method.[10] In view of the small amounts of potassium and calcium which are in blood, and the possibility of contamination (in the case of potassium even ammonium salts are to be avoided), vigorous chemical cleanliness of all syringes, needles, tubes, etc., which at any time came in contact with the samples was observed.

RESULTS AND DISCUSSION

The results of the analyses are shown in tables 1 to 3. The normal dairy cattle which received good diets containing mineral supplements did not at any time during the period of observation (about three years) show any of the symptoms which are associated with wheat poisoning. The average calcium content of the blood of these animals was 10.9 mg. per 100 cc., and the average potassium content was 16.7 mg. per 100 cc. The calcium contents varied from 10.4 to 11.9 mg. per 100 cc., while the potassium content varied from 12.9 to 20.9 mg. per 100 cc.

TABLE 1—The Serum Calcium and Plasma Potassium Levels of Normal Dairy Cattle

Exp. No.	Serum calcium mg./100cc.	Plasma potassium mg./100cc.	Ratio K/Ca
1	10.7	20.9	1.95
2	10.8	18.0	1.66
3	10.9	16.6	1.52
4	10.7	15.5	1.45
5	10.9	18.3	1.68
6	10.6	19.3	1.82
7	10.4	12.9	1.26
8	10.8	15.3	1.42
9	11.9	16.0	1.34
10	11.2	15.6	1.39
11	11.0	16.3	1.48
12	10.6	15.5	1.46
Ave.	10.9	16.7	1.53

The cattle which had been grazing wheat for two months did not show average blood calcium and potassium levels which were greatly different from those of the dairy cattle, but we feel that it is significant that the calcium values in these latter cases varied from a low normal figure of 10 mg. per 100 cc. down to a definitely low figure of 8.8 mg. per 100 cc. The lowest level in this series is not sufficiently low to be accompanied by observable symptoms of tetany, and no such symptoms were observed in this group. The average potassium content of the blood of these animals was above the normal for that of the dairy

cattle and ranged from normal values to slightly higher than normal values.

All of the animals which showed symptoms of tetany after grazing wheat had definitely low blood calcium values. Most of these animals showed potassium values

TABLE 2—The Serum Calcium and Plasma Potassium Levels of 10 Cows Which Had Been Grazing Wheat over Two Months and Receiving no Supplemental Rations

Exp. No.	Serum calcium mg./100cc.	Plasma potassium mg./100cc.	Ratio K/Ca
1	9.4	18.2	1.94
2	8.9	20.1	2.26
3	9.3	20.0	2.15
4	10.0	17.6	1.76
5	9.4	18.7	1.99
6	10.0	21.3	2.13
7	8.8	18.1	2.06
8	9.6	20.6	2.15
9	9.0	15.7	1.74
10	9.1	15.8	1.74
Ave.	9.3	18.6	2.00

which were either normal or below normal. All of these animals with 1 exception showed symptoms quite similar to parturient paresis. They even exhibited the typical S-shaped kink in the neck. The length of time which had elapsed since calving in these cases varied from one to eight months. These long periods of time would seem to eliminate the possibility of milk fever. The exception to these symptoms appeared in the example of case 1. This animal exhibited a wild behavior. She was able to get up when down, but walked with a stringhalted tetanic gait. She bellowed, chased the veterinarian, and vigorously resisted treatment. Her plasma potassium was definitely high and the K/Ca ratio was 4.03 compared with an average ratio of 2.73 for the other animals in this group, a ratio of 1.53 for normal animals, and a ratio of 2.00 for cattle grazing wheat, but exhibiting no symptoms of tetany. Therapy with calcium dextrose soon restored this animal to fairly normal behavior. About thirty minutes following therapy, her blood Ca was 14.8 per 100 cc., and the potassium showed a value of 24.8 mg. per 100 cc. The ratio at this time was 1.67, which is within the normal range.

We have found that cattle can be made to exhibit symptoms of wildness and tetany by elevating the blood potassium level by the oral administration of potassium chloride. These symptoms are observable when the ratio is about 3.35 with a normal serum calcium. If the blood calcium be lowered by intravenous administration of oxalates at the time the potassium is administered, symptoms of tetany have developed when the ratio has been as low as 2.62.[11] The higher ratio necessary to induce tetany by raising potassium levels without decreasing calcium levels is undoubtedly due to the fact that the ratio between ionized calcium and potassium is more significant than that between total calcium and potassium. Most procedures which would lower blood calcium undoubtedly affect the ionized calcium first.

It may be that this 1 animal showing symptoms of wildness simply represents an early stage of the condition shown by all

TABLE 3—The Serum Calcium and Plasma Potassium Levels of Cattle Exhibiting Symptoms of Tetany as a Result of Grazing Wheat

Exp. No.	Serum calcium mg./100cc.	Plasma potassium mg./100cc.	Ratio K/Ca
1	6.6	26.6	4.03
2	6.2	15.9	2.57
3	4.9	11.0	2.25
4	3.3	9.6	2.92
5	5.5	17.6	3.20
Ave.	5.3	16.1	3.04

the animals studied. It has been observed that, in early milk fever, cattle often exhibit symptoms such as those mentioned.

SUMMARY

1) The ratio of plasma potassium to serum calcium in normal dairy cattle was found to vary from 1.28 to 1.95 with an average value of 1.52.

2) This ratio for cattle grazing wheat and not showing symptoms of tetany varied from 1.73 to 2.26 and averaged 2.00.

3) In the case of cattle grazing wheat and showing symptoms of tetany, the ratio varied from 2.24 to 4.03 and averaged 3.04. In only 1 of these cases was the plasma potassium definitely high, and this animal exhibited more spectacular symptoms than the others.

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An Experiment on the Relationship of Diet to Hookworm Disease in Lambs

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RECENTLY, the writers[13] reported that growing lambs heavily infected experimentally with the common sheep hookworm (*Bunostomum trigonocephalum*) developed moderately severe anemia and gained considerably less weight than uninfected controls. Slight anemia and slight interference with normal gain were the maximum effects noted in lambs moderately or lightly infected.

These results were seemingly inharmonious with reports (Beller;[2] Velu and Balozet;[21] Daubney[7]) implicating natural infections, lighter than our heaviest experimental ones, as the cause of extreme anemia, cachexia, and fatalities among both lambs and older sheep in grazing flocks. Moreover, previous observations (Beller;[3] Ortlepp[15]) on experimentally infected sheep, though scanty and of somewhat doubtful significance, likewise were at variance with the failure of any of our test lambs to become seriously affected.

In view of these apparent discrepancies in results, we (*loc. cit.*) emphasized the possibility that the good diet fed our test lambs may have been chiefly responsible for the ability of these animals to cope so successfully with their infections. In advancing this suggestion, we were mindful of reports (Smillie;[19] Suárez;[20] Cruz;[5,6] Rhoads, Castle, Payne, and Lawson[18]) that the severity of hookworm disease in cases of human infection does not depend entirely, or even primarily, upon the number of worms harbored and that diet is an important factor which may modify the effects of infections of similar size.

To determine preliminarily whether diet affects the injuriousness of Bunostomum infections, a small-scale experiment was carried out in 1944 at the U. S. Department of Agriculture, Agricultural Research Center, Beltsville, Md. Two groups of lambs fed dissimilar diets were used. Two differing courses of exposure to infection were selected; each was tested in each dietary group. Both were similar to exposure levels which had resulted in heavy infections in our (*loc. cit.*) previous experiments. Moreover, intentionally, one of the diets selected for use in the present experiment was similar to the diet used in our previous work. The second diet chosen was intended to be roughly equivalent to poor pasturage in nutritive quality.

MATERIALS AND METHODS

Eight male lambs of mixed breeding, reared under conditions designed to minimize acquisition of parasites, were used. At the beginning of the experiment, they were about 2 to 2.5 months old and had been weaned only a few days. They were paired on the basis of age and weight, and each pair was housed in a concrete pen. Two pairs were fed a good diet and two pairs were fed a poor diet during the experiment. Until it began, alfalfa hay and grain had been available to all the lambs from birth.

The good diet was chopped U. S. No. 2 alfalfa hay and, in amounts indicated elsewhere, a daily ration of grain (usually 75 per cent whole oats, 17.5 per cent cracked yellow corn, and 7.5 per cent bran). The poor diet was chopped timothy and light clover hay; during the first seven days only, the lambs on this diet received a little grain also, as indicated elsewhere. Salt and water were available to both groups at all times.

The quantity of hay allowed the lambs was not restricted. Weighted amounts were placed daily, or more often as required, in a trough in each pen; the frequency of additions was regulated so that the lambs cleaned up the hay well, but at no time were the troughs empty. Coarse stems and dust, which the lambs repeatedly rejected, were weighed back periodically when the troughs were cleaned. If not badly trampled and soiled, hay strewn on the pen floor by the lambs was replaced in the

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Resigned (Neumayer) Sept. 28, 1945.

trough; that not so recovered was collected and its weight ascertained or carefully estimated. This procedure permitted calculation of the weight of hay actually consumed by each pair of lambs.

The pens in which the lambs were kept were cleaned on alternate days. Straw was used as bedding for the first few days. Since even the lambs on the good diet ate a little straw, shavings were substituted during the next week. Warm weather then set in and use of bedding was discontinued, as bare pen floors were advantageous for the purposes of the experiment.

TABLE I—Degree of Infection of Test Lambs

Infection Dates	Infective Bunostomum Larvae Placed on Skin* of—			
	Lambs on Poor Diet (Group 1 T)		Lambs on Good Diet (Group 2 T)	
	517	514	522	508
1944	*No.*	*No.*	*No.*	*No.*
Mar. 28	25,000	8,700	25,000	8,700
Mar. 30	16,000	.....	16,000	.....
Apr. 4	9,450	18,950	9,450	18,950
Apr. 6	.....	17,750	.....	17,750
Apr. 12	.....	9,750	.....	9,750
Apr. 15	.....	11,850	.....	11,850
Apr. 19	.....	23,150	.....	23,150
Apr. 24	.....	3,700	.....	3,700
Apr. 27	.....	16,500	.....	16,500
Apr. 29	.....	9,700	.....	9,700
May 2	.....	13,950	.....	13,950
May 5	.....	5,300	.....	5,300
	50,450	139,300	50,450	139,300

*Unavoidably, each lamb infected on the following dates received the following number of Strongyloides larvae: Apr. 6, 2,050; Apr. 12, 5,400; Apr. 15, 1,500; Apr. 19, 3,525; Apr. 24, 625; Apr. 27, 140; May 2, 130; May 5, 100.

The larvae used to infect the test lambs were obtained from cultures of the feces of two Bunostomum-infected sheep which had been reared free of parasites except Strongyloides and Eimeria. Their Bunostomum infections were experimentally induced. The cultures varied in age. However, the larvae used to infect different test lambs on the same day were invariably of common origin as they were derived from a single well-agitated suspension prepared by combining larvae freshly isolated from a group of cultures. Infections were induced by placing the larvae on the skin.

The general procedure and the laboratory methods used to follow the course of the infections and to determine their effects on the lambs were nearly the same as in our (*loc. cit.*) earlier experiments. Examinations of the blood and feces of the lambs were begun either one day before, or two days after, the test lambs were first exposed to infection; thereafter, these examinations were carried out at biweekly intervals.

In the figures in which certain of the experimental data are shown graphically, a long vertical line slightly to the right of the ordinate line signifies zero on the time scale, or the day larvae first were administered to the test lambs.

Experimental Data

Infection of Test Lambs

Feeding of the poor diet to one test pair (group 1T) and one control pair (group 1C) of lambs and of weighed amounts of the good diet to the remaining test pair (group 2T) and control pair (group 2C) was begun on March 28, 1944. Each test pair was first exposed to infection on this date also (table 1).

As shown by the table, (1) the lambs of groups 1T and 2T were dissimilarly exposed to infection; (2) lambs 517 (group 1T) and 522 (group 2T) were in every respect equally exposed, as were lambs 514 (group 1T) and 508 (group 2T); (3) lambs 517 and 522 were exposed to considerably less than half as many larvae as lambs 514 and 508; (4) infection of lambs 517 and 522 was completed within one week (three exposures); (5) infection of lambs 514 and 508 was spread over a 38-day period (11 exposures); (6) the initial exposure of lambs 517 and 522 was about three times as great as that of lambs 514 and 508; (7) within the first week, lambs 517 and 522 were exposed to about twice as many larvae as lambs 514 and 508.

Results of Occult Blood Tests

Poor Diet Group.—The feces of the test lambs were negative for blood on the fifteenth day of the experiment and in previous tests, but were positive on the eighteenth day. Therefore, loss of blood in their feces commenced between fifteen and eighteen days after infection. Intense reactions were obtained for lamb 517 earlier than for lamb 514. The feces of both were positive in four tests carried out during the life of lamb 517; in each, lamb 514's feces gave a less intense reaction than lamb 517's (fig. 1). The feces of the control lambs were consistently negative.

Good Diet Group.—The feces of the control lambs often gave weak positive reactions, since the benzidine test does not distinguish between hemoglobin and chlorophyll. The latter substance was abundant in the good diet. Such reactions also were obtained with the feces of the test lambs before their intestines possibly could have been invaded by developing larvae (Ortlepp[15]). Hence the interval between infection and the first appearance of blood in the

feces of these lambs could not be determined accurately.

However, the first significant reaction (*i.e.*, one more intense than any recorded for a control lamb) was obtained in the feces of lamb 522 twenty-two days after infection. This lamb's feces gave a significant reaction earlier than lamb 508's and

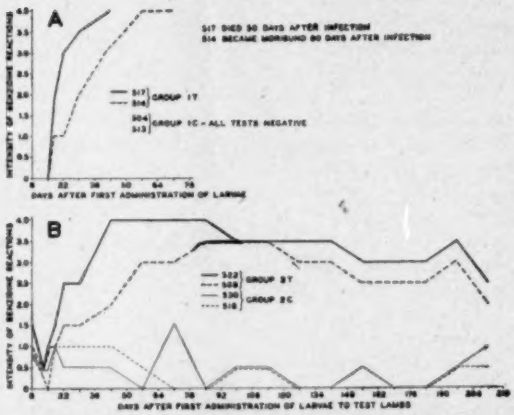

Fig. 1—Intensity of color reactions in benzidine tests for occult blood in feces of lambs. A—Reactions of feces of lambs of group 1 T; the feces of the controls were negative in all tests. B—Reactions of feces of lambs of group 2; presence of occult blood in the feces of lambs of group 2 T is indicated only by reactions more intense than any shown for the controls.

almost invariably, throughout the experiment, reacted more intensely than the feces of the latter lamb (fig. 1).

Comparison of Tests for the Two Groups.—Although the tests were not indicative of the absolute amounts of blood lost by the 4 infected lambs, the comparative reaction intensities shown graphically in figure 1† were harmonious with all other findings on the test lambs.

The following comparisons are considered significant: (1) reactions of high intensity were recorded for lamb 517 (group 1 T) earlier than for any other lamb, although reactions of maximum intensity were first recorded for lambs 517 and 522 (group 2 T) on the same day; (2) maximum reaction intensity was recorded earlier for lamb 522 (group 2 T) than for lamb 514 (group 1 T) and all reactions for these 2 lambs in the first seventy-one days of the experiment were roughly similar; however, diet probably influenced the reactions for lamb 522 to some degree; (3) reactions for lamb 508 (group 2 T) never reached the maximum intensity level recorded for the remaining test lambs.

DEVELOPMENT OF ANEMIA IN TEST LAMBS

Poor Diet Group.—The red-cell values of lamb 517 (group 1 T) remained within normal limits for the first seventeen days of

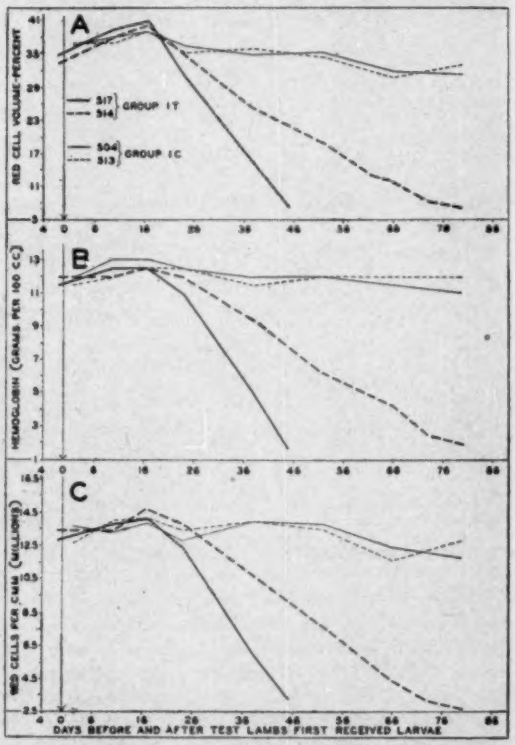

Fig. 2—(A-C) Hematocrit, hemoglobin, and red-cell count determinations on the blood of lambs of group 1.

the experiment, but in the next four weeks they declined in an essentially straight line to levels indicative of extreme anemia (fig. 2). On the forty-fifth day after infection, the red-cell values of this lamb were: volume per cent, 7.5; count, about 3,000,000 (3,362,000 in 1:100 dilution; 2,980,000 in 1:400 dilution); hemoglobin, 1.7‡ Gm. per 100 cc. Death resulted on the fiftieth day of the experiment.

†An explanation of the significance of the numerical expressions of reaction intensities used in the graph is given in our (*loc. cit.*) earlier paper.

‡Estimate: value obtained using a more concentrated blood dilution than is recommended for the hemoglobinometer employed.

TABLE 2—Summary of Counts of Total Leucocytes per Cubic Millimeter of Blood and of Results of Differential Counts in Smears of Blood in Lambs

Group No.	Lamb No.	Total and Differential Counts Made	*First Count	Total White Cells per Cmm.			Percentages of Indicated Types of White Cells and of Smudges Enumerated in Smears																	
							Lymphocytes			Neutrophils			Eosinophils			Monocytes			Basophils			Smudges		
		No.	1,000's	1,000's	1,000's	1,000's	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
				Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.	Max.	Min.	Av.
1 T	517	5	5.70	7.44	3.64	5.93	92.0	78.0	85.60	15.6	3.6	7.16	1.6	0.6	1.12	3.2	0.0	1.58	1.2	0.2	0.86	8.2	2.2	3.68
	514	9	10.80	10.80	4.12	6.41	94.2	70.6	81.32	26.0	3.6	11.80	10.2	0.0	2.39	2.8	0.4	1.36	1.2	0.0	0.41	5.0	0.5	2.74
(Av.)			8.25			6.17			83.46			9.48			1.76			1.47			0.64			3.21
1 C	513	8	7.70	10.48	2.48	7.16	89.4	77.2	85.35	19.2	4.4	9.95	0.8	0.0	0.25	3.2	0.4	1.58	2.0	0.0	0.55	4.8	0.4	2.33
	504	8	7.24	8.44	5.04	6.91	91.2	81.2	86.70	13.0	3.6	8.33	1.6	0.0	0.75	1.6	0.0	0.75	0.8	0.0	0.43	7.8	0.2	3.18
(Av.)			7.47			7.04			86.03			9.14			0.50			1.17			0.49			2.76
2 T	522	18	11.12	13.80	5.60	8.45	93.4	61.8	82.32	32.0	2.4	12.82	2.4	0.4	0.98	5.2	0.0	0.94	3.0	0.6	1.38	5.8	0.0	1.53
	508	18	8.85	9.04	4.20	7.15	91.6	64.0	79.84	30.8	6.6	19.34	3.2	0.0	1.04	4.8	0.0	1.41	2.2	0.2	1.29	1.8	0.0	0.58
(Av.)			9.98			7.80			81.08			16.08			1.01			1.18			1.34			1.06
2 C	518	18	8.00	8.76	4.36	7.28	93.2	62.8	78.02	35.0	5.4	18.54	2.9	0.0	0.67	2.4	0.0	0.61	1.2	0.0	0.56	4.2	0.0	1.51
	530	18	9.32	10.36	4.36	7.53	90.0	67.4	78.51	41.2	6.8	18.26	1.2	0.0	0.38	3.0	0.0	0.77	2.6	0.0	0.67	4.6	0.0	1.42
(Av.)			8.66			7.41			78.27			18.40			0.53			0.69			0.62			1.47

*Made on test lambs one day before infection; made on control lambs two days after start of experiment.

The red-cell values of lamb 514 (group 1 T) declined similarly, but less precipitously (fig. 2). This lamb also became extremely anemic. On the eightieth day of the experiment, it became comatose, its death appeared imminent, and it was slaughtered. On the previous day, its blood gave a hematocrit reading of 7 per cent, a red-cell count of 2,715,000, and a hemoglobin content of 2.0‡ Gm. per 100 cc.

The control lambs did not become anemic. Their red-cell values fluctuated within limits considered normal (fig. 2). The lowest hematocrit reading for either lamb in eight observations over a 79-day period was 31.0 per cent. Their red-cell values tended to decline slightly during the last month of observation, but it is doubtful that diet was responsible, since a similar tendency was apparent in the values for the well-fed controls (fig. 3). Moreover, this trend had not appeared when lamb 517 died. Therefore, it is clear that diet *per se* played no appreciable rôle in the production of the extreme anemia of the lambs of group 1 T.

Good Diet Group.—The lambs of group 2 T also became anemic, but only moderately so. Lamb 522 became more anemic than lamb 508 (fig. 3). Before the experiment was concluded, both had markedly, though not completely, recovered; lamb 508 had recovered to a greater extent than lamb 522. The control lambs did not become anemic, although their red-cell values fluctuated considerably.

The occurrence of anemia in lambs 522 and 508 was indicated by two facts: (1) each showed a markedly lower minimum value for each of the three indices than was recorded for either control lamb; (2) these minimums were attained as a result of progressive declines, which commenced about one month after the experiment began. Lamb 522 not only became more anemic than lamb 508, as indicated by the lower values recorded for it, but its anemia developed faster.

Type of Anemia.—Calculation of Wintrobe's[22] constants indicated that the anemia of lamb 517 (group 1 T) apparently was slightly microcytic. That of lamb 514

was normocytic. The anemia of both was normochromic, except perhaps shortly before death occurred. If the hemoglobin estimates‡ obtained terminally in these fatal cases are acceptable, hypochromia characterized the anemia in the final stage of the disease.

Although the anemia of lamb 508 (group 2 T) definitely was not microcytic, the more pronounced anemia of lamb 522 tended to be slightly so while it was in process of development. However, when the degree of anemia was greatest and in the recovery period, mean corpuscular volume tended to be above normal in both lambs. This fact is not construed as indicative of transition to a macrocytic anemia, because during the same period corpuscular volume tended to increase also in the control lambs (group 2 C). In neither lamb of group 2 T did hypochromia occur; in fact, during the recovery period, mean corpuscular hemoglobin often was greater than it had been early in the experiment, especially in lamb 522.

Thus, the anemia of Bunostomum infection was essentially normocytic, irrespective of diet or fatal or nonfatal course, and, except perhaps terminally in fatal infections, normochromic, so far as Wintrobe's constants revealed. This anemia is not, however, characterized by normality of the individual red cells. Erythrocytes in anemic blood, as observed in the hemocytometer, were far more variable in shape and size than those in the blood of the control lambs. The constants referred to are average values which, in our opinion, may mask compensating abnormalities among individual cells.

Relation Between Exposure to Infection, Anemia, and Diet

At both exposure levels tested, the greater degree of anemia appeared in the individual on the poor diet. However, within each dietary group, the test lamb which received the smaller number of larvae (table 1), the fewer administrations, the larger initial application, and the greater number of larvae in the first week of the experiment, was the one more severely affected (fig. 2, 3); in group 1 T, the ultimate severity of the anemia of the 2 lambs did not differ materially, but lamb 517 became anemic much more rapidly than lamb 514.

White Blood Cells, Infection, and Diet

Bunostomum infection did not markedly affect the total number of leucocytes per unit volume of circulating blood in either dietary group (table 2). It definitely did not cause leucocytosis. The differences in the dietary of the two groups did not materially affect the white-cell count, so far as was indicated by the averages of the counts for the two control pairs.

Differential counts (table 2) indicated, according to our interpretation, that in lambs on both diets infection was associated with slight eosinophilia and monocytosis. This is supported by comparison of the group average percentages of these types of cells and, with one exception, by comparison of the averages for the individual lambs. Maximum eosinophil and monocyte percentages were not in every case higher in infected than in control lambs. However, the highest observed percentage of eosinophils occurred in the blood of an infected lamb (514, group 1 T) as did the highest percentage of monocytes (lamb 522, group 2 T), and these percentages exceeded considerably the highest recorded for any control lamb.

Percentages of lymphocytes, neutrophils, and basophils were not demonstrably affected by infection (table 2). All lambs on the good diet had higher average neutrophil percentages than any lamb on the poor diet; however, full details (not shown in table 2) of the counts indicated that diet was not responsible for this fact. The difference in the dietary of the infected lambs of the two groups did not demonstrably affect their eosinophilic or monocytic response.

Significance of Results of Fecal Examinations

The Effective Pathogen.—One day after the experiment began and in all other examinations in the first fifty-two days, the feces of all lambs contained Strongyloides

eggs and coccidial oöcysts only (table 3; footnote *). The feces of all control lambs were free of Bunostomum eggs in all subsequent examinations also (table 3).

Hookworm eggs were first found in the feces of lambs 514, 522, and 508 fifty-three days after the experiment began. The feces of lamb 508 were negative the previous day. Therefore, the prepatent period of *B. trigonocephalum* was about fifty-three days in these infections. Counts of the Bunostomum eggs found in subsequent examinations (table 3) indicated that each of these 3 lambs harbored a significant infection.

The average intensity of coccidial infection, as indicated by the estimated density of coccidial oöcysts per coverslip examined (group averages, table 3), was similar in the test and control group on each diet. In no lamb were symptoms of coccidiosis observed.

Though the test pair of each dietary group had higher Strongyloides egg counts (group averages, table 3) than the control pair, no correlation existed between magnitude of Strongyloides egg count (individual averages, table 3) and anemia, or intensity of anemia (fig. 2 and 3). Moreover, we know of no experimental evidence of a correlation between Strongyloides infection and anemia in sheep.

Eggs of a few other kinds of helminths, not mentioned in table 3, occasionally were found in the feces of certain lambs. The sporadic appearance and small numbers of these eggs indicated that the corresponding extraneous infections were very light and hence unimportant.

These facts indicate that Bunostomum was the effective pathogen in the experiment.

Relation Between Bunostomum Egg Counts, Anemia, and Diet.—In group 2 T, egg-count levels and intensity of anemia were positively correlated. Lamb 522 had higher egg counts (table 3) and more intense anemia (fig. 3) than lamb 508. Egg-count levels, as well as intensity of anemia, were inversely related to the number of larvae received by these lambs.

In view of the findings at autopsy (table 4), similar relationships probably would have prevailed in group 1 T, had lamb 517 lived longer. As previously noted, it died fifty days after it first was exposed to infection. No hookworm eggs were found in its feces. These facts are of special incidental interest and practical importance.

TABLE 3—Results of Examinations of Feces of Experimental Lambs for Coccidial Oöcysts, and Bunostomum and Strongyloides Eggs

Group No.	Lamb No.	Bunostomum Eggs per Gram on Indicated Day After Start of Experiment*														Bunostomum Eggs /Gm./ Exam.†	Strongyloides Eggs /Gm./ Exam.‡	Density Index of Coccidial Oocysts§
		53	57	71	80	85	99	113	127	141	155	169	183	197	211			
		No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	
1 T	517‖	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	129	0.90
	514**	96	1,022	4,268	4,080	..	..	..	..	..	..	..	..	..	..	2,389	3,665	1.66
Av.																2,389	1,897	1.28
1 C	504**	..	0	0	..	..	..	..	..	..	..	..	..	..	..	0	105	0.90
	513**	..	0	0	..	..	..	..	..	..	..	..	..	..	..	0	266	1.57
Av.																0	185	1.24
2 T	522	6	174	3,268	..	6,200	3,040	4,520	2,600	2,920	3,880	2,123	2,132	2,400	2,268	2,733	295	1.14
	508	20	304	818	..	1,720	1,800	1,720	1,360	1,200	480	400	244	470	418	843	3,187	1.29
Av.																1,788	1,741	1.21
2 C	518	..	0	0	..	0	0	0	0	0	0	0	0	0	0	0	6	1.76
	530	..	0	0	..	0	0	0	0	0	0	0	0	0	0	0	8	1.18
Av.																0	7	1.47

*The feces of all lambs were negative for Bunostomum eggs 1, 15, 29, 43, and 50 days after the experiment began. The feces of lamb 508 also were examined and were negative fifty-two days after infection. †Per positive examination in the case of infected lambs. ‡Examinations were made each time feces were collected. §The density of the oocysts on a single coverslip was graded numerically (0 to 4) at each examination in accordance with arbitrary standards. The numerical grades so assigned were averaged to obtain the figures shown in this column. ‖Died fifty days after infection. **Slaughtered eighty days after infection.

Unquestionably, this lamb died of anemia caused by hookworms; yet fecal examination for worm eggs was completely valueless as a means of diagnosis of the cause of the anemia.

The four egg counts made on the feces of lamb 514 (group 1 T) were markedly higher than the first four counts for lamb 508 (group 2 T), and when lamb 514 had become extremely anemic, lamb 508 exhibited

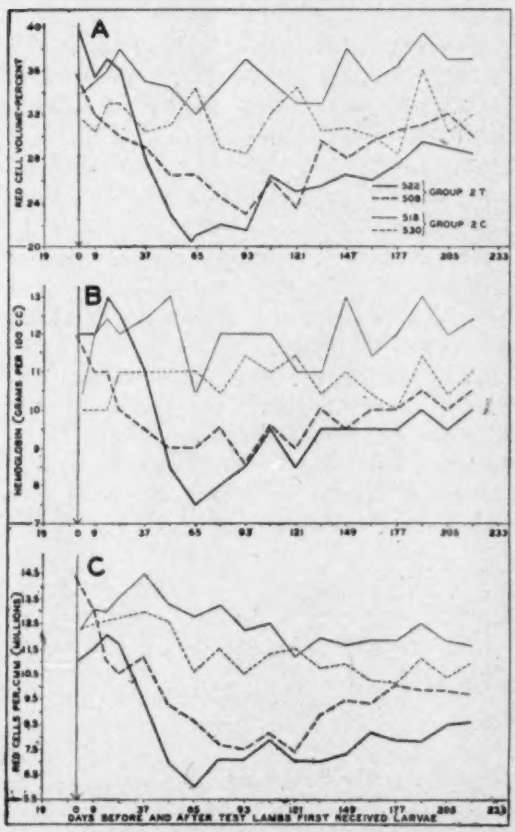

Fig. 3—(A-C) Hematocrit, hemoglobin, and red-cell count determinations on the blood of lambs of group 2.

only very slight anemia. However, in this instance higher egg count and greater injury were not associated with lesser exposure to infection but were coupled with inferior diet. These 2 lambs were equally exposed. Our interpretation§ of these circumstances is that percentage development was higher in lamb 514 than in lamb 508 and that its poor diet was the cause of its apparent acquisition of the greater number of worms. However, it should be mentioned, though the point will not be developed until later, that inferior hemopoietic ability due to poor diet probably was also a factor in the greater injury suffered by lamb 514, as compared to lamb 508.

The anemia of lamb 522 (group 2 T) likewise did not approach that of lamb 514 in severity. But in this instance greater injury was not associated with higher egg count. The averages of the first four counts for these lambs were nearly equal. This suggests§ that they harbored approximately equal infections. More direct evidence of similarity in the size of their infections will be presented later, and it will be pointed out that, because these infections were about equal, the dominant determinant of the greater injury produced in lamb 514, as compared with lamb 522, apparently was the depressing effect of poor diet on hemopoietic ability.

While the numbers of eggs in the feces of lambs 514, 522, and 508 were sharply increasing, the red-cell values of these lambs continued the downward trend which had commenced before the infections became patent. In the lambs which survived, these values showed a reversal of this trend and gradual improvement as egg counts declined from peak levels.

Relation Between Size of Infection, Anemia, and Diet

In group 2 T, lamb 522 harbored many more hookworms than lamb 508 when these animals were examined *post mortem* 224 days after infection (table 4). Thus, size of infection and intensity of anemia (fig. 3) were positively correlated in this group.

In group 1 T, lamb 517 was much more heavily infected at autopsy than lamb 514 (table 4) and the former lamb was more severely affected (fig. 2). Lamb 514 was examined *post mortem* thirty days later

§This interpretation of egg-count data may be questioned on the ground that dietary deficiencies have been reported to favor increased per female egg production (Foster and Cort[5, 9]). The data available to us on egg-worm ratios in our series of nine Bunostomum infections showed that these ratios varied considerably and justified only one conclusion, i.e., that if the egg count for 1 lamb was higher than for another, the former lamb, as a general rule, harbored more worms than the latter.

than lamb 517, and some worms undoubtedly were eliminated from its small intestine during that interval. However, the following indirect evidence indicates rather definitely that degree of anemia and size of infection were positively correlated in this group also.

The ratio of the number of worms recovered from lamb 508 to the number recovered from lamb 522 was 1:2.2 and the corresponding ratio in the case of lambs 514 and 517 was 1:2.8. Since lamb 508 was exposed to infection exactly as was lamb 514, and lamb 522 was exposed exactly as was lamb 517, the similarity of these ratios is evidence that in all probability lamb 514 at no time harbored as many worms as lamb 517. It also is interpreted as signifying (See also column 10, table 4) that percentage development was markedly affected by the degree or course of the exposure to infection (table 1). Both of these deductions are supported by the results of the occult blood tests performed. That percentage development apparently was independently affected by diet has been mentioned.

The number of hookworms recovered from lamb 522 (group 2 T) at the end of the experiment was equivalent to about 80 per cent of the number recovered 144 days earlier from lamb 514 (group 1 T). The probability that in this interval lamb 522 lost a considerable number of worms (footnote *, table 4) renders this percentage relationship a reasonably strong indication that lamb 514 was not appreciably more heavily infected than lamb 522 on the eightieth day of the experiment when the former lamb died. The radically greater injury suffered by it evidently, therefore, was not caused by a difference in worm burden, but was due in all probability to a comparatively inferior ability to compensate for blood loss due, in turn, to its comparatively poor diet.

The theoretical possibility that lamb 514 suffered greater early blood loss than lamb 522 must be conceded. It received the greater number of larvae. Therefore, the number of agamic worms which invaded its intestine, but failed to become established as adults, theoretically may have been greater than in the case of lamb 522. However, as already noted, the occult blood tests did not suggest appreciable difference in the blood loss of these lambs during the first seventy-one days of the experiment.

INFLUENCE OF INFECTION AND DIET ON WEIGHT GAINS AND FEED CONSUMPTION

Weight Gains.—The lambs of group 1 T lost weight during their respective periods of survival in the experiment, whereas the control lambs on the poor diet (group 1 C) gained a little weight (table 5). Data not included in table 5 indicated that in the period in which lamb 517 lost 5 lb., lamb

TABLE 4—Worms Recovered from Lambs at Necropsy

Group No.	Lamb No.	Date of Necropsy	Interval Between Final Admin. of Larvae & Necropsy	Interval Between Start of Exper. & Necropsy	Hookworms (*B. trigonocephalum*) Recovered*				Relation Between Total Larvae Admin. & Hookworms Recovered	Other Worms Recovered						
					M.	F.	Sex Undetermined (Head Ends)	Total		Nematodirus	Haemonchus	Strongyloides	Ostertagia	Cooperia	Trichostrongylus	Trichuris
		1944	*Days*	*Days*	*No.*	*No.*	*No.*	*No.*	*%*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*	*No.*
1 T	517	May 17	43	50	2,327	2,306	8	4,641	9.20	0	0	40	0	0	0	0
	514	June 16	42	80	845	818	0	1,663	1.19	0	0	1,466	0	0	5	1
1 C	504	June 17	†	81	0	0	0	0	†	0	0	37	0	1	0	0
	513	June 19	†	83	0	0	0	0	†	0	0	27	0	0	0	2
2 T	522	Nov. 7	217	224	632	699	0	1,331	2.64	0	0	49	1	1	0	0
	508	Nov. 7	186	224	282	330	0	612	0.44	0	0	2,399	0	0	0	0
2 C	518	Nov. 6	†	223	0	0	0	0	†	0	3	3	0	0	0	5
	530	Nov. 6	†	223	0	0	0	0	†	3	0	5	0	0	0	0

*Some of the hookworms from lambs 517 and 514 were immature. Worms in process of elimination from the host were found in the large intestine of 3 of the infected lambs as follows: lamb 517, 16 worms; lamb 514, 2 worms; lamb 508, 1 worm. †No larvae administered.

514 also lost this amount, whereas the average gain of the controls was about 2.5 lb.

The infected lambs on the good diet (group 2 T) gained appreciably in weight during the experiment (table 5). Data not included in table 5 showed that in the period during which lambs 517 and 514 lost 5 lb., lambs 508 and 522 gained 27 and 3 lb., respectively; in the period in which lamb 514 lost 10 lb., these group 2 T lambs gained 43 and 5 lb., respectively.

Thus, after equal exposure to infection, the lambs on the poor diet lost weight, while those on the good diet gained weight.

Though the writers are convinced that the weight gain of lamb 522 was adversely affected by infection, the data in table 5 cannot strictly be so interpreted because lamb 508 outgained the controls. However, lamb 508 developed the characteristics of a large breed not represented in the inheritance of the remaining lambs; moreover, it harbored somewhat less than half as many worms as lamb 522. Of far greater importance than the question of weight gains is the inference suggested by the full history of lamb 522, namely, that this inferior individual on a good diet was able to withstand an exposure to infection that proved fatal to an average lamb (517), which was fed a poor diet.

Feed Consumption.—The lambs of group 1 T showed no appreciable inappetence from March 28 to May 17 (table 6). Their loss of weight and the contrasting slight gain of the controls in this interval (table 5 and text) were not referable, therefore, to a difference in the feed consumption of the two pairs. However, in the thirty days after lamb 517 died, lamb 514 ate about 13 lb. less hay than the per-head consumption of the control pair. Its loss of weight in this interval was due no doubt mainly to failure to eat normally.

Thus, apparently in the poorly fed lambs infection affected both feed utilization and feed consumption, but not necessarily concurrently. Neither test lamb showed appreciable inappetence until it had become noticeably weak, and weakness was not marked until severe anemia was evident. The effect on feed consumption was, therefore, chiefly an indirect one.

In group 2, the control pair ate more hay during the experiment than the infected pair (table 6), but for each 100 lb. of alfalfa consumed gained slightly more weight. The infected pair ate about 90 per cent of the amount of hay eaten by the controls, and their gain (table 5) was about 87 per cent that of the controls. The weight of hay consumed per pound of gain during the experiment was 8.5 lb. for the controls and 8.8 lb. for the infected pair. Because of the inconsistent weight gains in this group (table 5), these facts are only suggestive of the possible effects of Bunostomum infection on the feed consumption and utilization of the well-fed lambs.

It is of considerable incidental interest that, from March 28 to June 17, the control lambs on the good diet consumed (table 6)

TABLE 5—Gain or Loss in Weight of Lambs During and Before Experiment*

Group No.	Lamb No.	Age at Start of Exper.	Wt. at Birth	Wt. at Start of Exper.	Wt. Gained Before Exper. Began	Wt. at Last Weighing Before Death or Slaughter	Wt. Gain (+) Or Loss (−) in Exper.	Period in Which Gain or Loss in Wt. Accumulated	Weekly Rate of Gain (+) or Loss (−) in Wt.	
									In Pre-Exper. Period	In Exper.
		Weeks	Lb.	Lb.	Lb.	Lb.	Lb.	Weeks	Lb.	Lb.
1T	517	8.43	11.50	44.50	33.00	39.00	−5.50	7.57	+3.91	−0.73
	514	8.71	12.75	47.50	34.75	37.50	−10.00	11.86	+3.99	−0.84
1C	513	8.43	12.00	48.25	36.25	54.00	+5.75	12.00	+4.30	+0.48
	504	9.86	10.25	40.00	29.75	46.50	+6.50	12.00	+3.02	+0.54
2T	522	7.86	10.75	34.00	23.25	71.00	+37.00	32.00	+2.96	+1.16
	508	9.43	13.50	45.00	31.50	129.50	+84.50	32.00	+3.34	+2.64
2C	518	8.29	11.75	48.00	36.25	116.00	+68.00	32.00	+4.37	+2.12
	530	7.29	11.50	42.00	31.50	113.50	+71.50	32.00	+4.32	+2.23

* For the purposes of this table, March 25, the date of the last weighing before larvae were administered to the test lambs, is taken as initiating the period of the experiment.

4.8 lb. of afalfa (plus 0.9 lb. of grain) per pound gain in weight, while the controls on the poor diet consumed 18.6 lb. of timothy (plus an extremely small amount of grain) per pound gain in weight. The greater economy of the good diet is obvious.

TERMINAL SYMPTOMS OF FATAL BUNOSTOMIASIS

During the last few days of its life, lamb 517 became noticeably lethargic as evidenced by a disposition to lie down, by failure to respond normally to stimuli, and by failure to eat. When prodded into rising, it stumbled. Finally, it was unable to arise, became comatose, and died. As previously noted, lamb 514 behaved similarly. Beller,[2] and Velu and Balozet[21] described kindred symptoms as part of the syndrome of bunostomiasis. These investigators regarded them as nervous manifestations. Beller[3] claimed the degree of anemia in the cases observed by him did not suffice to explain such behavior and he postulated that these symptoms were caused by a hookworm toxin acting on the central nervous system. We consider, however, that anemia was the basic cause of all symptoms observed in our fatal cases.

DISCUSSION AND CONCLUSIONS

Although this experiment was on a small scale, its results were impressive because both well-fed lambs survived and largely recovered from the effects of infections resulting from exposure to exactly the same numbers of larvae that induced infections which were fatal to both poorly fed lambs. Moreover, in our (*loc. cit.*) previous experiments, 2 heavily infected, well-fed lambs, though somewhat older, were affected about as were the well-fed lambs in the present experiment.‖ One harbored at autopsy approximately as many worms as were found in lamb 514 of the present experiment. The second, which received a course of larval administrations not differing materially from that given lamb 514, yielded an even greater number of worms.

From all of these facts, we conclude that diet actually was the factor responsible for the vastly different disease picture observed in the two test groups of the present experiment.

Beyond this, the results are susceptible of less definitive interpretation, but, for reasons already explained, details of the data strongly suggest, in our opinion, that equal exposure to infection led to far more drastic effects in the poorly fed than in the well-fed lambs because (1) a higher percentage of the larvae administered developed in the former lambs, and because

‖While we have not produced a fatal infection in any of 8 lambs fed alfalfa hay and grain, despite exposure percutaneously to about 5,000 to 600,000 larvae, the following case should be noted: On March 22, 1944, when 9.5 weeks old, lamb 501, not involved in an experiment, was exposed to 10,500 larvae and placed in a pen with 2 other Bunostomum-infected sheep. The diet was alfalfa hay only. On May 29, lamb 501 died of extreme anemia as shown by a hematocrit reading of 4.5 per cent on blood withdrawn just before death occurred. The number of hookworms recovered was 2,441. However, the lamb was also heavily infested with sheep keds (*Melophagus ovinus*) which suck blood. Moreover, while its diet obviously was qualitatively intermediate between the two diets used in the present experiment, feeding was not supervised; although the amount of hay provided presumably was adequate, possibly the lamb was forced to subsist largely on the leavings of the 2 older sheep.

TABLE 6—Feed Consumption of Lambs

Group No.	Daily Grain Ration /Group in Indicated Periods				Total Grain Consumption /Group	Kind of Hay Fed	Weight of Hay Consumed /Head in Indicated Periods			Total Wt. of Hay Consumed /Head While on Exper.	Total Gain (+) or Loss (—) in Body Wt. /Head While on Exper.	Hay Consumed /Lb. Body Wt.	
	Mar. 28 to Apr. 4	Apr. 4 to May 8	May 8 to May 22	May 22 to Nov. 4			Mar. 28 to May 17	May 17 to June 17	June 17 to Nov. 4			Gained	Lost
	Lb.	Lb.	Lb.	Lb.	Lb.		Lb.	Lb.	Lb.	Lb.	Lb.	Lb.	Lb.
1 T	0.25	*	*	*	1.75	Timothy	61.25	38.00†	‡	99.25§	—10.00†		9.9†‖
1 C	0.25	*	*	*	1.75	Timothy	63.50	50.75	‡	114.25	+6.13	18.6	..
2 T	0.50	0.50	0.75	1.00	198.00	Alfalfa	71.63	69.75	392.38	533.75	+60.75	8.8**	..
2 C	0.50	0.50	0.75	1.00	198.00	Alfalfa	78.88	72.50	443.00	594.38	+69.75	8.5††	..

*Received no grain after April 3. †Lamb 514 (lamb 517 died on May17). ‡So far as this group was concerned, the experiment was terminated on June 17. §Lamb 514 (based on its consumption from May 17 to June 17 and the per head consumption for the previous period indicated). ‖The figure for lamb 517 was 11.1 lb. (based on the per head consumption during its life). **For the interval from the start of the experiment to June 17, i.e., the period covered by the data for the poor diet groups, the figure was 5.9 lb. ††For the interval from the start of the experiment to June 17, the figure was 4.8 lb.

(2) the ability of these lambs to compensate for blood loss was inferior to that of the well-fed lambs.

So far as we are aware, the literature on hookworm disease contains no report of experimental results that clearly are inconsistent with the claim that diet exercised a twofold influence in this experiment. The view that diet affected ability to compensate for blood loss is, however, less well supported than the view that it affected resistance.

That deficient diets usually lower the resistance of the host to infection by hookworms and by other helminths is amply attested by a considerable body of experimental evidence, much of which was mentioned by Ackert[1] in a fairly recent review of the subject. Although dogs, cats, chickens, and laboratory animals are the hosts that have been studied most, it also has been shown (Fraser and Robertson;[10] Clunies Ross and Gordon;[4] Kauzal;[12] Fraser, Thompson, Robertson, and George[11]) that a poor diet increases the susceptibility of sheep to infection with Haemonchus and some of the other common helminth parasites of this host.

The reports referred to in the introductory section of this paper, according to which diet is a factor controlling the degree of anemia produced in cases of human hookworm infection, seem to be thoroughly acceptable, although the evidence presented in them perhaps cannot be characterized as strictly experimental in nature.

Although hookworm disease in dogs has been extensively studied experimentally, attention has been concentrated on the relationships between diet and resistance to infection, and we have not encountered a single report purporting to show that a certain number of hookworms caused less anemia in well-nourished than in poorly nourished dogs. In fact, we cannot cite any completely acceptable experimental report showing that diet affects the ability of domestic or laboratory animals to combat the effects of helminthic parasitism, as distinguished from ability to cope with the invaders themselves. Though actual testing of the point by animal experimentation seems to have been neglected, apparently few, if any, investigators doubt that diet does affect the former ability in the case of hookworm anemia as it is practically an *a priori* certainty that certain deficiencies in diet must lessen the host's ability to generate blood elements.

It is true that Otto and Kerr[16] stated, with reference to *Ancylostoma caninum* infections in dogs, that acquired immunity apparently is by far "the most important natural means of host control of the infection" and conceded only that an animal with "a hyperactive and almost inexhaustible" hemopoietic system "might" be able to compensate for the hemorrhage caused by the infection. Moreover, Otto and Landsberg[17] explained the survival of well-nourished and the death of poorly nourished dogs, all given the same number of infective larvae, as due to the failure of the latter dogs to develop sufficient immunity to protect themselves. However, they also stated that erythropoietic insufficiency or failure occurred in the dogs on the deficient diet and they believed that this diet was inadequate for the maintenance of hyperactive erythropoiesis because of its low iron and protein content. Other dietary factors mentioned by them as essential for hemoglobin regeneration were copper and the vitamin B complex. We suspect that calcium and possibly still other dietary elements also may be important in hookworm disease.

From calculations based on the average proportions of alfalfa hay, corn, bran, and oats per pound of feed actually consumed by our well-fed lambs and on analytical data presented by Morrison,[14] it appears that the lambs fed timothy hay received per pound of feed consumed an amount of calcium equal to about 22 per cent of that received by the well-fed lambs, and that the corresponding percentages of digestible proteins and iron were about 28 and 59, respectively. Timothy hay is also inferior to alfalfa hay as a source of vitamins A, D, E, and G. Moreover, the lambs on the poor diet ate less feed per day than those on the good diet.

SUMMARY

At the USDA Agricultural Research Center at Beltsville, Md., in 1944, a pair of lambs on a good diet (alfalfa hay and grain) and a pair on a poor diet (chiefly timothy hay) were infected experimentally with *Bunostomum trigonocephalum*. One member of each pair was exposed to a total of about 50,000 infective larvae, and the second member of each pair was exposed to a total of about 139,000 infective larvae of that hookworm species. The larvae were administered serially by skin, commencing when the lambs were about 2 months old. Administration of the larger number of larvae was spread over a 38-day period, but the smaller number was given within one week.

Extreme anemia developed in both lambs on the poor diet and caused their death. The one exposed to 50,000 larvae died fifty days after infection and yielded 4,641 hookworms. Its dietary mate was slaughtered *in extremis* eighty days after infection and yielded 1,663 hookworms. Thus, anemia developed more rapidly in the former than in the latter lamb. A pair of uninfected controls on the same diet showed no anemia in eighty days.

The infected pair on the good diet also became anemic, but only moderately so. They not only survived, but about seven months after infection their red-cell values had regained, or nearly regained, normality. The minimum hematocrit reading recorded for the lamb exposed to 50,000 larvae, which yielded 1,331 hookworms when slaughtered 224 days after infection, was 20.75 per cent. The minimum recorded for its dietary mate, which at the same time yielded 612 hookworms, was 23.0 per cent. Anemia developed more rapidly in the former than in the latter lamb. A control pair on the same diet did not become anemic.

Obviously, within each dietary group, difference in exposure to infection affected the intensity of the subsequently induced anemia, speed of development, as well as minimum red-cell values ultimately recorded, being regarded as an index of intensity. However, diet was regarded as the determinant of the differing severity of the hookworm disease induced in the lambs equally exposed to infection.

Details of the data were considered to suggest that diet affected both susceptibility to infection and ability to compensate for blood loss. Compared with the good diet, the poor diet was deficient in protein, iron, calcium, and certain vitamins.

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The Influence of Sulfamerazine Upon the Incidence of Avian Lymphomatosis

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THE EFFECTIVENESS of sulfonamides for the control and/or prevention of lymphomatosis has received relatively little study. Asplin[1] found that most sulfonamides in common use were effective against a virus-like disease in young chickens which he suggested might be an acute form of fowl paralysis (neurolymphomatosis). The virus disease in question was demonstrated[2,3] by the inoculation of a suspension of tissue from typical cases of neurolymphomatosis. Scott *et al.*[4] reported apparent recovery of clinical cases of neurolymphomatosis with sulfanilamide treatment; however, residual evidence of the disease was found in 5 out of 6 cases. Burmester[5] found that sulfamerazine, when fed at a maximum level of 4 Gm. per pound of total feed consumed, had no preventive or curative effect on the two transplantable lymphoid tumor strains he used.

It is believed that chickens may become infected with lymphomatosis at an early age. Experiments for determining the effectiveness of sulfonamides against this disease complex must be designed so that a relatively high concentration of the chemotherapeutic agent is available soon after hatching and is maintained for a considerable period of time. Sulfamerazine was chosen since it was reported by Severens, Roberts, and Card[6] to be better tolerated by chickens than most other sulfonamides when a high blood level concentration is maintained.

MATERIALS AND METHODS

The birds used were pedigreed Single-Comb White Leghorns from the Laboratory flock. Waters and Bywaters[7] described the origin and management of the foundation flock. In this study a single hatch of chicks from stock highly susceptible to lymphomatosis was used. The families of chickens were divided as nearly as possible between experimental group A and control group B to equalize genetic variations of the families.

Immediately after hatching, the groups, consisting of 100 birds each, were placed in different battery brooders in the same room. Group A chickens were placed on a diet of chick mash containing 1 per cent sulfamerazine. At the end of the fifth week the concentration of sulfamerazine was reduced to 0.5 per cent owing to the development of toxic symptoms. Uniform mixing of the sulfamerazine and mash was assured by the use of a feed mixer. The chick mash used for the group B diet and for mixing with sulfamerazine was the same as that routinely used at this laboratory. Both groups were given free access to fresh water and their respective diets.

When 44 days of age, both groups of chickens were transferred from the battery brooders to a floor pen in the laying house. The floor pen was divided into two parts by common chicken wire, and each pen thus formed had its own feed hoppers and water founts. The attendant worked in both pens without changing clothes, including shoes, when caring for one group or the other. In this way, the environment was essentially the same for both groups.

All male birds were destroyed at the end of the fifth week due to limited space. Thus, the two experimental lots of chickens were reduced to 37 females in the sulfamerazine group (A) and 46 females in the control group (B).

At 300 days the experiment was terminated and all remaining birds were destroyed and necropsy was conducted. All diagnoses were based on macroscopic examination.

RESULTS AND DISCUSSION

At the end of the fourth week toxic symptoms were noted in the sulfamerazine group. The symptoms observed were indicative of retarded growth and of a slight polyneuritis. Swollen combs in the group A male birds were noted a few days later as further indication of toxicity. At the end of five weeks, toxic manifestations were so marked that the concentration of sulfamerazine in

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the feed was reduced from 1.0 to 0.5 per cent. The revised diet was tolerated, the neuritis disappeared, and the growth rate increased noticeably. The average weight gain of group A was actually greater than that of group B during the last hundred days of the experiment. Birds of group A averaged 1,954.6 Gm. and group B averaged 1,889.06 Gm. at the termination of the experiment.

Mattis *et al.*[8] showed that sulfamerazine did not materially affect 32-day old chickens when fed at a concentration of 1 per cent in mash for fourteen days. Welch *et al.*[9] found that chickens averaging 770 Gm. in weight and given 0.25 gr. of sulfamerazine every twelve hours gained weight. The same birds remained free from symptoms of peripheral polyneuritis for an experimental period of twelve days. Burmester[5] found that sulfamerazine was apparently nontoxic to birds 2, 3, and 46 days of age when fed continuously at a level of 4 Gm. per pound of feed for as long as twenty-six days.

The results of this experiment were similar to those of Mattis *et al.*,[8] Welch *et al.*,[9] and Burmester[5] for the concurring period of the experiment; however, over a longer period of time it was found that gains in weight were retarded and toxic symptoms developed.

There was no apparent effect upon egg production and normal egg-shell formation. Genest and Bernard[10] reported that sulfamerazine has a negligible effect upon egg-shell quality as compared with unsubstituted sulfonamides and sulfanilamide, which cause the shell to be thin and rough.

During the tenth week, an outbreak of coccidiosis occurred in the control group resulting in the death of 3 birds. The sulfamerazine group showed no evidence of coccidiosis.

Lymphomatosis was first diagnosed in the control group on the forty-seventh day and in the sulfamerazine group on the 150th day, both cases being of the neural type. The incidence of lymphomatosis in the 37 birds of the sulfamerazine group was 6 (16.2%), 4 of the neural type and 2 visceral. In the 46 controls, 7 (15.2%) were positive, all of the neural type.

Mortality other than from lymphomatosis was due to cannibalism, impaction of the proventriculus, prolapse of the oviduct and, in addition, group B had coccidiosis. In group A, mortality from these miscellaneous causes was 5.4 per cent as compared with 21.7 per cent for group B.

SUMMARY AND CONCLUSIONS

1) Two groups of 100 birds each, from stock highly susceptible to lymphomatosis, were used for the experiment. Subsequently, the males were discarded, leaving 37 females in the sulfamerazine group (A) and 46 females in the control group (B). Immediately after hatching, the experimental group was fed a chick mash diet containing 1.0 per cent sulfamerazine. Toxicity was noted at the end of the fourth week, and the concentration of sulfamerazine was reduced to 0.5 per cent at the end of the fifth week, after which toxic symptoms subsided.

2) There was no apparent effect upon egg production and normal egg-shell formation in the sulfamerazine group.

3) A mild outbreak of coccidiosis occurred in the control group but not in the sulfamerazine group. The two groups were housed in the same room separated by a chicken-wire partition.

4) The incidence of lymphomatosis in the sulfamerazine fed group was 16.2 per cent of 37 birds, while 15.2 per cent of the control group of 46 birds developed gross lesions. Mortality from all causes was 21.6 per cent of 37 birds in the sulfamerazine group compared with 36.9 per cent of 46 birds in the control group.

5) Sulfamerazine did not prevent the development of naturally occurring avian lymphomatosis.

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Influence of the Route of Inoculation of Newcastle Disease Virus on Selective Infection of the Embryonating Egg

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THE INFLUENCE of the route of inoculation of Newcastle disease [avian pneumoencephalitis] virus on hemagglutination titers at several sites in the embryonating egg was reported by Burnet in 1934.[5] Now that the hemagglutination test is being used quite widely in the diagnosis of this disease, the effect of the route of the virus inoculation on embryo lethal titers as well as on hemagglutination titers was studied. The correlation of these two methods for measuring infection was also investigated.

Burnet used three methods of inoculation —upon the chorioallantoic membrane, into the amnionic, and into the allantoic chambers. Following inoculation upon the chorioallantoic membrane, hemagglutination titers after death of the embryos were 1:20 for allantoic fluid and 1:2 for amnionic fluid. When inoculation was into the amnionic chamber, the allantoic fluid gave a titer of 1:200 and the amnionic fluid 1:800. Inoculation into the allantoic chamber resulted in an allantoic fluid titer of 1:320 and an amnionic fluid titer of 1:10. Brandly and coworkers[3] reported that allantoamnionic fluid from embryonating eggs infected by inoculation upon the chorioallantoic membrane was not hemagglutinative, or only slightly so. When the allantoic, amnionic, or intravenous routes were employed, the fluids from fatally infected eggs were invariably quite active.

EXPERIMENTAL PROCEDURE

The present study consisted of plotting the rate of development of hemagglutinins in embryonating eggs at six-hour intervals following inoculation of 11-day-old embryos and of checking a portion of these materials to determine the highest dilution which would produce at least a 50 per cent mortality of embryos, *i. e.*, the $l.d._{50}$ titers. Four routes of inoculation— (1) intravenous, (2) upon the chorioallantoic membrane, (3) into the allantoic chamber, and (4) into the yolk sac— were tried.

A strain of virus (NJ, K & D) isolated in New Jersey,[1] subsequently carried in eggs, and having a titer in allantoamnionic fluid of 10^{-9} for embryos was used throughout the experiment. The standard inoculum consisted of one lot of pooled allantoamnionic fluid apportioned into tubes for each separate preparation of inoculum and subsequently held at —35 C.

Eleven-day embryonating eggs from a Newcastle disease susceptible Leghorn flock were inoculated with 0.1 cc. of a virus dilution containing 10^6 lethal doses. The $l.d._{50}$ titer of the virus was rechecked at each inoculation by the route of inoculation employed and found to be the same, regardless of the route.

A modification of the intravenous method[2] was employed. A narrow rectangle of shell over the selected vein was removed and a drop of mineral oil placed onto the shell membranes to make them transparent. A 27-gauge needle attached to a 0.25-cc. Luer-type, tuberculin syringe was introduced into the visible vein in the direction of the blood flow, the hub resting on the shell, and amounts up to 0.1 cc. inoculated. The chorioallantoic membrane inoculation followed in general the methods of Woodruff and Goodpasture[6] and Burnet.[4] The membrane was dropped just prior to inoculation and the shell sealed with cellulose tape. The yolk-sac[7] and the allantoic chamber[8] inoculations were performed according to conventional methods.

One route of inoculation was studied at a time. Incubation was in a 37 to 38 C. forced-draft incubator. Following inoculation, five eggs were selected at random every six hours over a 48-hour period and removed from the incubator, chilled, harvested, and representative material from each pooled. The eggs were chilled at 4 to 6 C. for several hours to facilitate the harvest of clear fluids.[6] Ten-cc. syringes carrying needle-screen-adapters were used to draw off separately the three egg fluids, *i. e.*, the allantoic, the amnionic, and yolk-sac fluids. Relatively good separation of the allantoic and amnionic fluids as indicated both by the physical appearance of the fluids and their sharply differing titers was accomplished with the screen-tipped needles. The extraembryonic membranes, *i. e.*, the amnionic, the chorioallantoic, and yolk sac, were separated, washed three times in saline, dried on a

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sterile blotter, and weighed. The lungs of the embryo were treated in a like manner. These weighed tissues were then ground separately in TenBroeck-type grinders in a measured amount of saline to make a 1:5 suspension and centrifuged for twenty minutes at 2,000 r.p.m. before hemagglutinative or embryo l.d.$_{50}$ titers were determined.

The procedure for the hemagglutination test described by Brandly and coworkers[3] was followed throughout.

EXPERIMENTAL RESULTS

The order and time of appearance of demonstrable hemagglutinins was distinctive for each route (fig. 1, 2, 3, and 4). Replicate experiments gave no significant variations. Hemagglutinins developed most rapidly in eggs inoculated by the intravenous route. Titers of 1:80 were demonstrable at twelve hours in amnionic membrane and chorioallantoic membrane suspensions. Embryos inoculated by the yolk-sac and allantoic chamber route produced positive hemagglutination titers first at eighteen hours. With the yolk-sac route the titer of the amnionic membrane suspension at eighteen hours was 1:80, that for the allantoic fluid and chorioallantoic membrane suspensions, 1:10. Following the allantoic chamber route the allantoic fluid titers was 1:20 at eighteen hours and that of the amnionic membrane suspension, 1:5. Materials from embryos inoculated upon the chorioallantoic membrane failed to show hemagglutination titers until twenty-four hours after inoculation.

The extraembryonic membrane became infected first by all four routes of inoculation. The amnionic membrane was the first site to show infection by the yolk-sac, allantoic chamber, and intravenous routes, and the second site by the chorioallantoic membrane route. The first tissue invaded in the chorioallantoic membrane inoculated eggs and also in those intravenously inoculated was the chorioallantoic membrane. The yolk-sac membrane was quickly infected when the inoculation was by that route. The last sites to show the presence of hemagglutinins were the yolk-sac fluid and the embryonic lung suspensions.

In almost all instances, hemagglutination

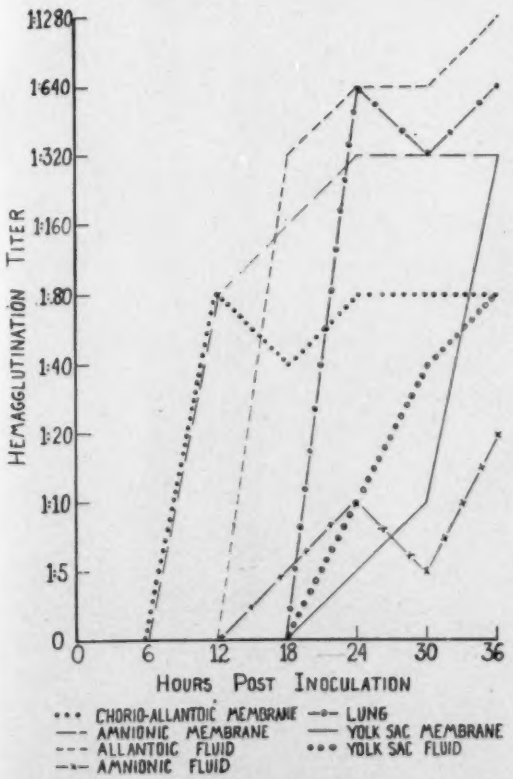

Fig. 1—Selective infection of Newcastle virus by the intravenous route.

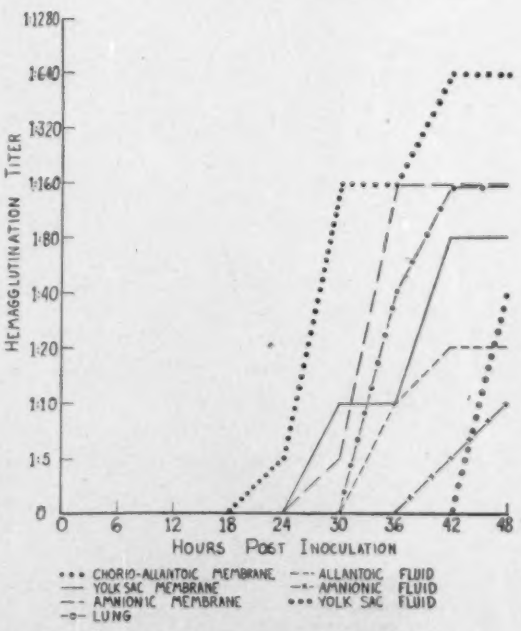

Fig. 2—Selective infection of Newcastle virus by the chorioallantoic route.

titers showed a progressive increase from the first appearance of the hemagglutinins until the death of the embryo, although in sites where rapid development occurs early there was a gradual leveling off of titer before death ensued. The hemagglutination titers at the time of death confirmed the observations of Burnet[5] and of Brandly and coworkers.[3] The effect of the four routes of inoculation on allantoic fluid harvested after the death of the embryo is presented in figure 5. Fluids from eggs inoculated by intravenous and allantoic chamber routes gave titers satisfactory for routine hemagglutination work. Those from yolk-sac inoculated eggs were only fair; fluids from chorioallantoic membrane inoculated eggs were too low for significant diagnostic work. As a rule, amnionic fluids gave lower titers than allantoic fluids and yolk fluids still lower. Titers of 1:320 or better were found several times for allantoic fluid, amnionic membrane, chorioallantoic membrane, yolk-sac membrane, and lung suspensions (fig. 1 to 4).

The route of inoculation had an influence on the death time of the embryo. Intravenous and yolk-sac inoculated eggs were dead at thirty-six hours. A few of the embryos included in the intravenous group lived up to forty-eight hours, possibly due to difficulties in technique associated with the somewhat subnormal development of the allantoic veins, and the resultant allantoic chamber rather than intravenous inoculation. Embryos inoculated into the allantoic chamber and upon the chorioallantoic membrane died between forty-two and forty-eight hours after inoculation. In all instances, except in some of the aforementioned intravenously inoculated embryos, the range of individual death times of the embryos was relatively narrow, all deaths having occurred within a six-hour period.

In preliminary tests a normal hemagglutinin for adult chicken red blood cells was found in the supernatant of centrifuged eleven- to thirteen-day yolk-sac membrane suspensions which so closely resembled the virus hemagglutinin in its agglutinative activity as to be indistinguishable from it. The presence of the normal hem-

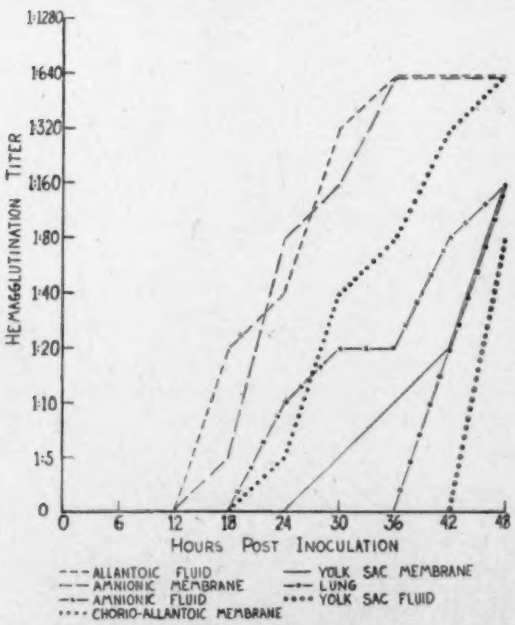

Fig. 3—Selective infection of Newcastle virus by the allantoic chamber route.

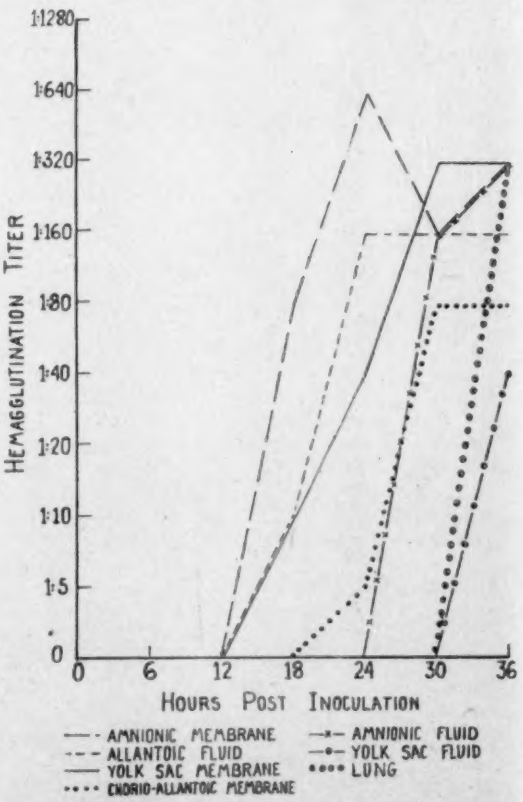

Fig. 4—Selective infection of Newcastle virus by the yolk-sac route.

agglutinin in both normal and Newcastle infected yolk-sac membranes completely "masked" the virus hemagglutinative activity and made the demonstration of the latter impossible. The normal hemagglutinin gave titers averaging about 1:320, falling in one or two instances to indistinguishable titers and rising a few times to 1:1,280. Subsequently, it was demonstrated that normal chicken serum, added in the same manner and amount as is the immune serum in the virus hemagglutination-inhibition test, inhibited the normal yolk-sac hemagglutination reaction, and "unmasked" the virus hemagglutinin so that it could be demonstrated (fig. 6). Newcastle immune serum inhibited both the normal and virus hemagglutinins. All yolk-sac membrane titers, given in figures 1 to 4, were end-points observed after the normal yolk-sac hemagglutinin was inhibited by normal chicken serum.

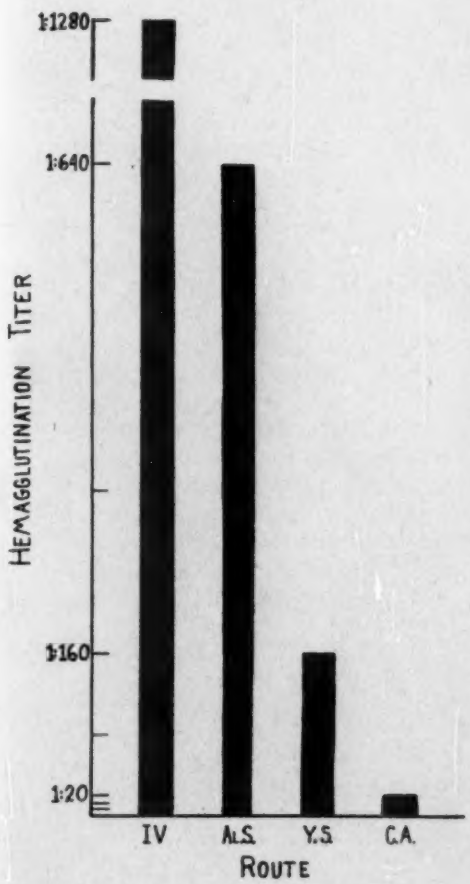

Fig. 5—Hemagglutination titers of allantoic fluids after death of the embryo—comparison of four routes of inoculation.

The same suspensions and fluids used in making the hemagglutination determinations were used to find the embryo $l.d._{50}$ titers. One tenth cubic centimeter quantities of tenfold serial dilutions were inoculated into eleven-day embryonating eggs intra-allantoically, four eggs being used per dilution. The eggs were then incubated at 37.5 C. and candled daily. The end-point was determined as the highest dilution in which at least half of the embryos died, *i.e.*, $l.d._{50}$. The limited number of eggs available for such titrations prevented plotting a curve similar to the hemagglutinin development curve to show the increases of virus as expressed by the $l.d._{50}$ titer of the various sites.

The embryo $l.d._{50}$ titration is much more sensitive as an indicator of the presence of Newcastle virus than is the hemagglutination test. It was easily possible to show the presence of virus as early as six hours following inoculation, *e.g.*, allantoic fluid from eggs inoculated by the intravenous route had a titer of 10^{-2} at six hours, 10^{-3} at twelve hours, and 10^{-7} at eighteen hours. The hemagglutination test, however, was found positive first at eighteen hours after inoculation. A pattern of relation between hemagglutinins and embryo $l.d._{50}$ titers became apparent after a number of such tests were run (fig. 7).

Virus suspensions having an embryo $l.d._{50}$ titer of 10^{-5} or less failed to produce demonstrable hemagglutination. Those with embryo $l.d._{50}$ titers of 10^{-6} or higher produced positive hemagglutination titers that increased in general relation to the rise in $l.d._{50}$ titers. Suspensions with an embryo titer of 10^{-6} had corresponding hemagglutinin titers ranging from undiluted or 1:5 to 1:160. With an embryo titer of 10^{-8} the agglutination range was from 1:160 to 1:1,280. The type of suspension—embryonic membrane, tissue, or fluid—had no effect on the result.

CONCLUSIONS AND SUMMARY*

Newcastle virus hemagglutinin activity and the embryo l.d.$_{50}$ titer are two expressions of the amount of virus present in a given amount of infected material. The sensitivity of these two tests is greatly different. The embryonating egg is very susceptible to Newcastle virus, 50 per cent of deaths having been induced by one millionth of the amount of virus demonstrable by the hemagglutination method here employed. The relatively low sensitivity of the hemagglutination test for Newcastle virus does not reduce the value of the test since embryos routinely inoculated by the allantoic and intravenous routes invariably produce high titer allantoic fluids about the time of death. The use of allantoic fluids from embryos inoculated upon the chorioallantoic membrane is not indicated for hemagglutination work.

A correlation was demonstrated between Newcastle virus hemagglutination and embryo l.d.$_{50}$ titers, the latter detecting smaller quantities of virus. The influence of four routes of inoculation on development of virus hemagglutinins and of virus as evaluated by the l.d.$_{50}$ titer for embryos was shown for seven egg materials. The route of inoculation did not yield any differences in infectivity titers as determined by l.d.$_{50}$ values.

A hemagglutinin for chicken red blood cells was demonstrated in both normal and infected yolk-sac membranes, and a means of inhibiting it is presented.

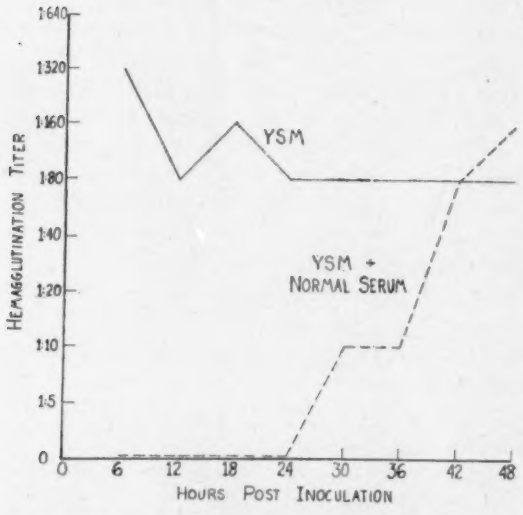

Fig. 6—Demonstration and inhibition of normal yolk-sac membrane hemagglutinins.

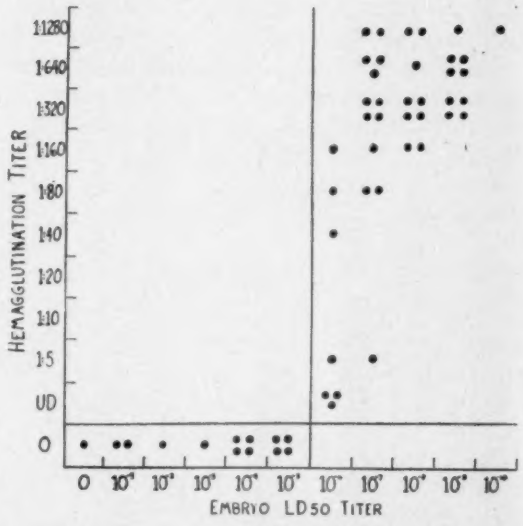

Fig. 7—Correlation of hemagglutinins and embryo l.d.$_{50}$ titers of Newcastle virus.

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*Since completion of these studies, there has come to our attention the work of Burnet and Stone (Austral. J. Exptl. Biol. and M. Sci., 24, (1946): 1-8 and 9-13) in which normal hemagglutinins for some samples of chicken cells have been demonstrated with alcoholic and aqueous extract suspensions of normal chorioallantoic membranes and normal liver tissue of 14- to 16-day chicken embryos as well as of various mammalian tissues.

Morphogenesis of the Parathyroid Glands of Chicken Embryos

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THE ENTODERMAL origin of the parathyroid glands of the birds was first shown by Verdun.[8] He believed they were derived from part of the third and fourth branchial pouches.

Sun[7] stated that, at the eighth day of incubation, the parathyroid tissue is cordlike in arrangement. He reported that, during the last part of the incubation period, the glands become similar in appearance to the adult glands; at this time, they are cordlike and the cell groups are separated by connective tissue. Since this characteristic cordlike structure remains throughout the incubation period, Sun doubted that the parathyroid glands have any function in the embryo.

Forsyth[4] has described the thyroid and parathyroid glands of several species of birds. The gross anatomy and histologic structure of the parathyroids are briefly discussed. Frequently, this author observed thyroid glands which contained varying amounts of parathyroid tissue. In all cases, where both types of structures were present, no line of separation between the two could be found. Parathyroids attached to the posterior pole of the thyroid were often found to be continuous with the thyroid parenchyma. Small accessory glands situated near the thyroid often contained thyroid and parathyroid tissue.

MATERIALS AND METHODS

All embryos and chickens used in this study were of a pure single-comb, White Leghorn stock maintained by the Department of Poultry Husbandry at Iowa State College. Throughout the study, the fertile eggs were obtained from the same group of hens.

A series of 420 embryos, ranging from twelve hours of incubation to hatching, was used for the morphologic studies.

The parathyroid glands were fixed in either Zenker's, Bouin's, or Susa's fluid. All tissues were embedded in Altman's paraffin mixture. The sections were cut at 6 to 8 μ in thickness.

The stains most frequently employed were hematoxylin and eosin. For differentiating the connective tissue in the parathyroid glands, the Zenker-fixed material was stained with Mallory's connective tissue stain.

Observations.—On the postero-lateral surface of each thyroid lobe, at 148 hours of incubation, a small group of cells appeared to be separating from the thyroid lobe by an invasion of connective tissue (fig. 1). These cells, after detachment from the thyroid primordium, became the parathyroid primordium.

At 165 hours, the parathyroid primordia had become completely detached, and had migrated 75 μ posteriorly from the thyroid lobes. From these primordia, another group of cells was detached by invading connective tissue. This latter group of cells developed into the small posterior parathyroid lobe, while the former developed into the large anterior parathyroid lobe (fig. 2).

The anterior lobe of the parathyroid at 213 hours was crescent-shaped and surrounded by undifferentiated mesenchymal cells. The smaller posterior lobe was located 50 μ posterior to the anterior lobe. The posterior lobe was a small mass of cells which took a very deep basophilic stain with hematoxylin (fig. 5).

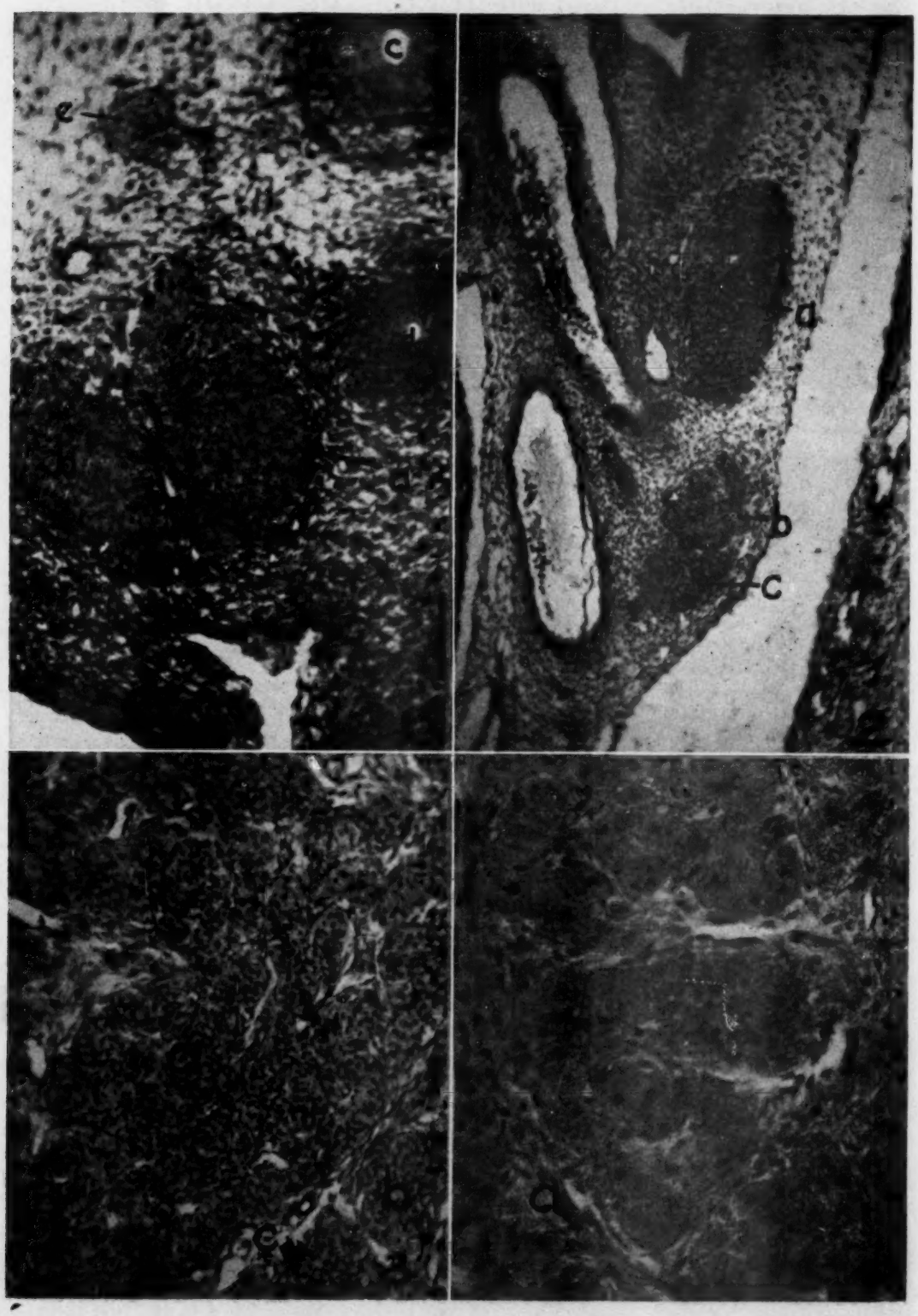
At 228 hours, the anterior lobe showed an increase in size as compared with the previous observation period. It was semicircular and gave off branches of cell cords. Mesenchyma, containing capillaries, could be seen between the branching cords of cells. Morphologically, the cellular elements resembled those of the developing thyroid gland. The posterior lobe consisted of several small, branching cell cords; no blood vessels were present in the mesenchyma between the cords.

The cell cords, at 240 hours, continued to increase in size and to vary considerably in shape. The cell cytoplasm was diminishing; the nuclei were smaller and took more basophilic stain than those cells of the thyroid at this period.

A portion of the data presented in a thesis submitted to the graduate faculty of Iowa State College in partial fulfillment of the requirements for the Ph.D. degree, March, 1942.

Assistant professor, Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus.

Sincere thanks are extended to Dr. H. L. Foust, chairman of the Department of Veterinary Anatomy, Iowa State College, Ames, for his counsel and generosity in supplying materials and laboratory equipment; to Dr. M. Lois Calhoun, Department of Veterinary Anatomy, Michigan State College, East Lansing, for her assistance in preparing photomicrographs.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At 252 and 260 hours of development, the cell cords showed much mitotic activity and were more closely grouped. The vascular supply increased in the surrounding mesenchyma.

The cell cords were closely packed at 272 and 284 hours of development. The mesenchyma surrounding the cords had become compressed within the lobes. The cell nuclei were round to oval and the cytoplasm distinctly acidophilic in reaction (fig. 6).

The parathyroid lobes at 307 hours were composed of closely packed, convoluted epithelial cell cords. The cytoplasm of many of the cells contained what seemed to be a chromophobic colloid, located near the nucleus. These cells appeared to be similar to those of the thyroid just before definite follicular formation has occurred.

At 319 hours, two types of cells differentiated on the basis of staining appeared in the parathyroid lobes. One was a large, rather clear cell, staining lightly with eosin and possessing a large, poorly chromatic nucleus. This cell type decreased in number as development progressed and, at 375 and 388 hours, possessed a nongranular cytoplasm and a light-staining vesicular nucleus. By 400 hours of development, this cell type was seen to occur in very limited numbers. The second type of cell contained a small, highly chromatic nucleus and cytoplasm which stained deeply acidophilic. This cell type predominated in the gland and, at 375 and 388 hours, possessed a finely granular cytoplasm. At 400 hours of development, this cell type became less acidophilic, and its nucleus was slightly vesicular. Its nucleus was smaller and less vesicular than that of the first cell type (fig. 4).

A third cell type could be recognized in the parenchyma of the parathyroids at 400 hours. This type occurred singly or in groups and contained large amounts of granular cytoplasm which stained intensely with acid dyes. The nucleus was much smaller than in the other two cell types described above, and more deeply basophilic.

The first and third cell types described above varied markedly in numbers as development progressed. At 412 and 438 hours, both of these cell types were greater in numbers than during previous observation periods.

At 450 hours, the first cell type was rarely seen and only a few of the third cell type were present. The second cell type described was predominant in the glands. At this time, fine connective tissue trabeculae penetrated the parenchyma from the glandular capsule (fig. 7).

At 462 hours, a few of the first cell type were present, either singly or in groups. The third cell type had increased in number and occurred singly or in groups throughout the entire parenchyma. Little difference now existed in size between the three cell types.

At 474 and 498 hours, the glandular tissue was extremely compact, reducing the size of the sinusoidal capillaries. The cell cords were two cells in thickness. Both lobes were in close proximity, and the reticular fibers of one lobe extended into the other (fig. 3).

DISCUSSION

The fact that the parathyroid glands are derived from visceral pouches 3 and 4 was not verified by this investigation. Lillie[3] does not describe the parathyroid glands, but he does mention epithelial vestiges from visceral pouches 3, 4, and 5. Verdun[9] found that the ventral surface of pouch 3 developed an epithelial node whose structure and evolution was similar to that of the parathyroid glands of mammals.

The parathyroid glands developed along with the thyroid glands. The parathyroid primordia evaginated with the thyroid from the floor of the pharynx and migrated posteriorly with the thyroid primordia. At 148 hours, a lobe, lateral to each thyroid, was pinched off (fig. 1). At 165 hours, a small group of cells was separated from the anterior lobe of the parathyroid. These cells became the small posterior lobe of the parathyroid (fig. 2).

The parathyroid glands did not develop as a spherical mass of cells. Following

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Fig. 1—A sagittal section taken from a 148-hour chicken embryo. On the postero-lateral surface of the thyroid lobe, a group of cells is being detached and will become the anterior lobe of the parathyroid gland. The parathyroid primordium becomes detached from the thyroid lobe as a result of mesenchymal connective tissue invasion into the thyroid tissue. Hematoxylin and eosin. x 200. (a) Thyroid lobe; (b) parathyroid primordium; (c) esophagus; (d) trachea; (e) thymic primordium.

Fig. 2—A sagittal section taken from a 165-hour chicken embryo. At this time, the parathyroid primordium has migrated posteriorly from the thyroid lobe and is detaching a group of cells which becomes the small posterior lobe of the parathyroid gland. Hematoxylin and eosin. x100. (a) Thyroid lobe; (b) parathyroid primordium which will become the anterior lobe; (c) parathyroid primordium which will become the posterior lobe.

Fig. 3—A section of parathyroid tissue taken from a 474-hour chicken embryo. Hematoxylin and eosin x 200. (a) Anterior lobe; (b) posterior lobe; (c) connective tissue separating the two lobes.

Fig. 4—A section of parathyroid tissue taken from a 388-hour chicken embryo. Hematoxylin and eosin. x 400. (a) Connective tissue capsule.

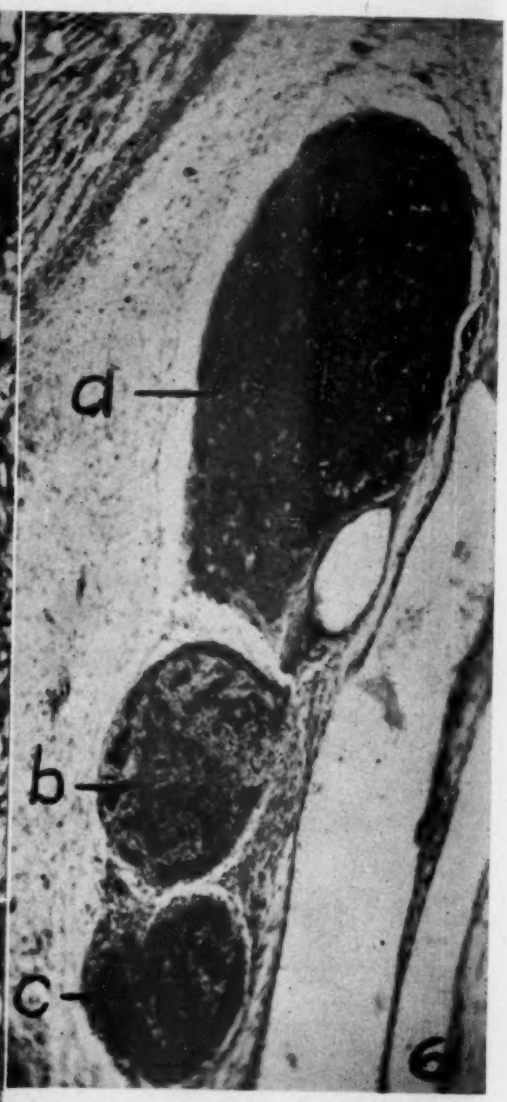

Fig. 5—A sagittal section taken from a 213-hour chicken embryo. The small posterior lobe of the parathyroid gland has migrated posteriorly since its detachment from the anterior lobe parathyroid primordium. Hematoxylin and eosin. x 200. (a) Thyroid lobe; (b) anterior lobe of parathyroid gland; (c) posterior lobe of parathyroid gland.

Fig. 6—A sagittal section taken from a 272-hour chicken embryo. Observe the relations of the thyroid and parathyroid lobes. The convoluted cordlike growth of the parathyroid lobes is distinct. Hematoxylin and eosin. x 100. (a) Thyroid lobe; (b) anterior lobe of parathyroid; (c) posterior lobe of parathyroid.

Fig. 7—A section of parathyroid tissue taken from a 450-hour chicken embryo. Hematoxylin and eosin. x 400.

differentiation, the cells formed a crescent-shaped epithelial cord, which branched as the gland developed (fig. 5, 6). Nonidez and Goodale[4] stated that the cords of parathyroid cells, when fully developed, are directed toward the center of the gland. This interpretation is incorrect, since the cords of parathyroid cells developed away from the center of the gland.

The lobes of the parathyroids developed unequally in size. The first mass of cells, detached from the thyroid tissue, became the larger of the two lobes. The small group of cells detached from the anterior lobe became the smaller, posterior lobe. The larger, anterior parathyroid lobes were usually found almost in contact with the posterior pole of the thyroid lobes. Nonidez and Goodale[4] mentioned instances in which this position was reversed. No cases of such reversal of position were observed during this investigation.

In addition to the main parathyroid glands, there were occasionally found masses of accessory parathyroid tissue occupying varying positions. A similar observation was made by Nonidez and Goodale.[4] Accessory parathyroid tissue and thymic tissue have been found embedded in the posterior lobes of the thyroid, and in the ultimobranchial bodies which are situated in the loose connective tissue in the space between the carotid artery and jugular vein. Verdun[5] has shown that the ultimobranchial bodies formerly regarded as the "lateral" lobes of the thyroid, arose from a fifth pair of rudimentary pharyngeal pouches. From the material used in this investigation, it appears that the ultimobranchial bodies developed from the floor of visceral pouch 4. Much variation occurred in the development of these bodies. In some embryos, they were well developed, while in others, only a small mass of cells could be found. Without the aid of serial sections, many of the ultimobranchial bodies would go unnoticed.

The presence of thymic and parathyroid tissues in the same organ is explained by the fact that, as the thyroid and parathyroid cells lost connection with the pharyngeal epithelium, some of the cells from the third and fourth visceral pouches were carried posteriorly in the migratory process and became differentiated later in embryonic life. The third and fourth visceral pouches gave rise to the thymus.

Nonidez and Goodale[4] explained the presence of thymic and parathyroid tissues in the same organ by the fact that, since the ultimobranchial bodies represented remnants of rudimentary pharyngeal pouches, a complete separation of the thymic and parathyroid anlagen, such as normally occurred in the third and fourth pairs of pouches at earlier stages of development, had not occurred.

The presence of parathyroid tissue in the ultimobranchial body would mean that a few cells from the parathyroid anlagen were carried down with the fourth visceral pouch when it separated from the pharynx. That is, as the thyroid and parathyroid anlagen separated from the pharynx, a few cells from the parathyroid anlagen remained in contact with the fourth visceral pouch, since this pouch did not lose connection with the pharynx until 134 hours of incubation. The thyroid and parathyroids lose connection with the pharynx as early as ninety-six hours.

Similarly, the presence of parathyroid tissue embedded in the thyroid parenchyma would indicate that, as the parathyroid anlagen was detached from the thyroid anlagen, a few cells were lost and later became differentiated.

The three cell types present in the parathyroids at 400 hours of development may be similar to those described for mammals. The first type is known in mammals as the clear chief cell, the second, as a small, dark chief cell (Petersen;[6] Getzowa;[7] Noodt[8]), and the third type, as a large, bright oxyphilic cell (Welsh[9]). The possible functional interrelationship of the parathyroid cells and the other endocrine glands of the developing chicken embryo has previously been reported (Venzke[3]).

Summary

The morphogenesis of the parathyroid glands of 420 developing chicken embryos was studied by means of serial, sagittal, transverse, and frontal sections, taken at approximately twelve-hour intervals from the twelfth hour of incubation until hatching.

The parathyroid primordium evaginated with that of the thyroid from the floor of

the pharynx, migrated posteriorly, and became separated from the thyroid primordium as a lateral lobe at 148 hours of development. This was the anterior parathyroid lobe. After 165 hours of development, the posterior lobe of the parathyroid gland became detached from the anterior lobe.

The parathyroid glands developed into cords of cells radiating from a central, cresent-shaped epithelial cell cord.

Accessory parathyroid tissue was occasionally embedded in the posterior lobes of the thymus, under the capsule of the thyroid with thymic tissue, and in the ultimobranchial bodies.

Three types of cells, based on cellular structure and staining characteristics, were demonstrable in the parathyroid glands at 400 hours of development.

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The Response of Some Mammals to Newcastle Virus

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PRELIMINARY observations of a California strain of Newcastle [avian pneumoencephalitis] virus adapted to the Syrian hamster showed that after the eighth intracerebral passage the strain could be carried without difficulty in the species, producing 100 per cent mortality.[1]

DISTRIBUTION OF VIRUS

To determine the distribution of Newcastle virus in the blood, liver, spleen, kidney, lung, and brain of hamsters of the twelfth and of the fourteenth intracerebral passage, these tissues were injected into 11-day embryonated White Leghorn eggs. Each organ was removed aseptically, ground with alundum, and diluted to a 10 per cent suspension with physiologic saline solution. One-tenth of one cubic centimeter of serial dilutions, ranging from 10^{-1} to 10^{-5}, of the blood and of each organ, was inoculated into the allantoic sac of each of three eggs. Newcastle infected allantoic fluid of known titer was used as a control in the titrations.

These tests demonstrated that the virus was present in the brain but not in the other organs or in the blood. In the titrations of the twelfth hamster passage, the brain suspension titrated 10^{-4}, while that of the fourteenth hamster passage titrated 10^{-3}.

NEUTRALIZATION TEST

A 10 per cent suspension of hamster brain, sixteenth passage, which titrated 10^{-4} in 10-day embryonated eggs, was used for a neutralization test with the hamster as the test host. Those hamsters inoculated intracerebrally with a mixture of hamster-adapted Newcastle virus and chicken Newcastle convalescent serum showed no symptoms of Newcastle virus infection within a period of ten days. Serum taken from the hamsters on the tenth day showed no neutralizing antibodies when tested in embryonated eggs. Hamsters inoculated intracerebrally with a mixture of normal chicken serum and hamster-adapted Newcastle virus succumbed with symptoms typical of Newcastle virus infection on the third and fourth days following inoculation. A group of normal hamsters used as controls was inoculated with sixteenth passage hamster-adapted virus, and all died on the third and fourth days of Newcastle virus infection. Thus it was proved that chicken Newcastle convalescent serum neutralized the hamster-adapted California strain of Newcastle virus, while the normal chicken serum failed to do so.

In further experimental trials with hamster-adapted Newcastle virus of the sixteenth passage, normal hamsters of approximately 40 Gm. each were given intranasally 0.1 cc. of a 10 per cent brain suspension. On the eighth and ninth days after exposure, all showed symptoms similar to those seen in hamsters exposed to Newcastle disease virus by intracerebral injection. At autopsy no gross lesions were observed in the lungs or other organs. Emulsions of lungs and of brain from these hamsters were instilled intranasally into young hamsters in an effort to continue propagation of the virus by this route. All hamsters exposed to the second intranasal passage remained normal in appearance during an observation period of fifteen days. The presence of virus in the above suspensions was shown by the reactions in embryonated eggs.

One-half cubic centimeter of a 10 per cent suspension of sixteenth passage hamster-adapted Newcastle virus was injected intracerebrally into each of 3 young guinea pigs and each of 2 young albino rabbits. On the

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third day after inoculation, 1 of the guinea pigs showed irritability and malaise, the same as hamsters similarly injected. This was followed by a rapidly spreading paralysis. Another guinea pig appeared slightly abnormal on the fifth day but recovered, while the third remained normal during an observation period of twelve days. Both rabbits failed to show symptoms. The guinea pig which showed symptoms of Newcastle virus infection was sacrificed while moribund. The brain was removed aseptically, ground with alundum, and diluted with physiologic saline solution to a 10 per cent suspension. Of this suspension, 0.5 cc. was passed intracerebrally into 3 young normal guinea pigs as a second guinea pig passage. The guinea pigs so exposed failed to develop symptoms of Newcastle virus infection. The presence of virus in the brain suspension was shown by the reactions in hamsters injected intracerebrally.

TABLE 1—Passage of Hamster-Adapted Newcastle Virus in Mice

Passage No.	No. animals inoc. Ic.	Animals paralyzed, moribund, or dead No.	%	No. days after inoc. paralysis occurred			
1	6	3	50	4	5	6	
2	5	4	80	2	5	8	10
3	4	1	25	5			
4	6	4	66.6	2	6	8	8
5	4	0	0				
5	5	0	0				
5	2	0	0				

Ic. = Intracerebrally.

When 0.03 cc. of a 10 per cent brain suspension of the eighth hamster-adapted passage of Newcastle virus was injected intracerebrally into each of 6, 3-week-old white Swiss mice, 3 of them developed symptoms of Newcastle virus infection, as described for hamsters and a guinea pig. While the mice were moribund, they were sacrificed for passage. By this procedure it was possible to make four successive intracerebral implantations. Attempts at a fifth passage were unsuccessful on three trials. Table 1 indicates a more detailed account of these passages. The presence of virus in the mouse brain suspension used for the fourth passage was shown also by the reactions in hamsters injected intracerebrally and in embryonated eggs. The presence of virus in the mouse brain suspension used for the inoculation of mice in fifth passages was not proved because embryonated eggs and hamsters were not immediately available.

The results are similar to those reported by Brandly and coworkers,[2] working with egg-propagated Newcastle virus, in that the hamster-adapted virus was not successfully passed in mice beyond the fourth passage. Toxic effects of intracerebral injection of allantoic fluid containing active human and porcine influenza virus, as noted by Henle and Henle,[3,4] did not seem to enter into the results, because the percentage of symptoms and deaths followed no definite downward trend with successive passages, and the appearance of symptoms and the deaths did not occur in a uniformly short time. The intracerebral route of exposure seems to preclude the possibility of complications, such as obtained in the intratracheal injections of Newcastle virus as made by Burnet.[5]

TABLE 2

No. of monkey	Route of exposure	Inoculum	Dose	Results
1	In.	Egg-adapted virus of 10^{-3} titer in embryonated eggs.	1 cc. of 10^{-1}	No symptoms.
2	Ic.		0.3 cc. of 10^{-1}	No symptoms.
3	In.	Hamster-adapted virus of 21st passage of 10^{-4} titer in embryonated eggs.	1 cc. of 10^{-1}	
4	Ic.		0.3 cc. of 10^{-1}	Moribund 18th day. Destroyed.

In. = Intranasal. Ic. = Intracerebral.

Because of the severe neurotropic symptoms produced by both egg-adapted and hamster-adapted California strain of Newcastle virus in the animals mentioned, it was believed that the monkey might be susceptible. Four young rhesus monkeys,

about 4 lb. each, were exposed intranasally and intracerebrally, as indicated in table 2.

Serum obtained from the monkeys prior to exposure showed no natural neutralizing antibodies for Newcastle virus when tested by the egg-embryo neutralization test. Monkeys 1 and 2, inoculated with the egg-adapted virus, and monkey 3, inoculated intranasally with the hamster-adapted virus, showed no symptoms. Monkey 4, inoculated intracerebrally with the hamster-adapted virus, was normal until the sixteenth day after inoculation, when it became very restless and nervous and held its head back against the shoulder. On the seventeenth day this monkey was able to sit erect but was dull and sleepy, frequently allowing the head to rest on the chest. At intervals the head was carried backward, so that it almost touched the back. The animal was able to move about but did so very listlessly. The whole attitude was one of progressive weakness and nervous depression. On the eighteenth day this monkey was completely paralyzed and was sacrificed. On autopsy the surface of the brain was congested, while all other organs appeared normal. Serum from the surviving monkeys taken on the twentieth day after inoculation showed no neutralizing antibodies.

A 10 per cent suspension of the infected monkey brain was passed intracerebrally to 3 young hamsters. Seven days later 1 of these showed symptoms of Newcastle virus infection.

Histologic studies have not been carried out on all materials collected. These examinations are to be made, and a later report will be published. Such studies as have been made on brain tissue from affected animals have shown congestion and hemorrhage with no indication of cellular infiltration.

Symptoms have been constant in hamsters infected by intracerebral exposure with embryo-propagated Newcastle virus and with hamster-adapted Newcastle virus through numerous passages. Similar symptoms have been shown by other species which became infected from the injection of hamster-adapted Newcastle virus. The possibility of the presence of other viruses as the basis for the disease in hamsters seems remote, in that chickens which become infected from the injection of hamster-adapted virus show typical symptoms of the original virus disease. The results in mice and guinea pigs are in line with those of other investigators working with Newcastle virus.

SUMMARY

After the eighth intracerebral passage, the hamster-adapted Newcastle virus was passed successively in hamsters through more than 50 series by the same inoculation route. By means of tests in embryonated eggs, it was shown that the virus was concentrated in the brain, but it was not demonstrated in other vital organs or the blood of sacrificed moribund hamsters. It was of interest to find that the hamster-adapted Newcastle neutralization test in hamsters correlated with the neutralization test in embryonated eggs. Newcastle hamster-adapted virus produced irritability and malaise[1] without producing lung lesions, when introduced intranasally into hamsters, but further passage was not accomplished. Newcastle hamster-adapted virus failed to infect rabbits but induced infection in mice and guinea pigs when injected intracerebrally; but continued passage in these species was not possible. A rhesus monkey, inoculated intracerebrally with the hamster-adapted virus, developed Newcastle disease symptoms; but a monkey inoculated intranasally with the same virus, and monkeys inoculated intranasally and intracerebrally with the egg-adapted virus, did not develop Newcastle virus infection. A time interval varying from forty-eight hours to sixteen days from injection to first symptoms or death was seen in the affected experimental animals, regardless of inoculum or route of exposure. In most pathogenicity tests, virus was shown present in the inoculum by the injection of embryonated chicken eggs or other species. In a small number of examinations histopathologic lesions were congestion and hemorrhage, with no indication of cellular infiltration. The possibility of a virus other than that

of Newcastle disease being carried in hamsters appears remote.

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Hemoglobin Concentrations in the Blood of Normal and Estrogen-Treated Turkeys

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IN CLINICAL dosages, diethyl stilbestrol appears to produce no marked pathology in the blood of man.[1,2,3,4] A slight anemia has been noted in rhesus monkeys following daily intramuscular injections of 10 mg. of diethyl stilbestrol,[5] but large doses of estradiol dipropionate did not produce this effect.[6] In rats, 1.88 mg. of diethyl stilbestrol absorbed daily from subcutaneous pellet implants produced only minor effects on the blood cells as judged from smears of peripheral blood and bone marrow after three weeks of treatment.[7] Administered by stomach tube, however, 2.14 mg. daily produced "a hypoplasia of the marrow with a depression of all the cellular elements of the peripheral blood."[8] Similar results have been reported following subcutaneous injections of considerably smaller amounts of diethyl stilbestrol[3,9] and of estradiol benzoate.[10] The latter substance has been reported to depress erythropoietic activity in rats made anemic by hemorrhage.[11] In mice, much smaller dosages (slightly over 2 μg. daily for thirty-six weeks) by intramuscular injection failed to produce an anemia of more than questionable significance.[12] Thus the species summarized above appear to be relatively resistant to estrogen-induced anemia except at high dosage levels. In dogs, however, anemia is readily induced by injection of 5 mg. diethyl stilbestrol daily.[3,5,13,14,15]

The effect of estrogens on the blood of birds has been incompletely studied. Bankowski has summarized the literature up to 1942 on normal hemoglobin concentrations in the blood of chickens.[16] On the blood of turkeys, only the papers of Rhian, Wilson, and Moxon,[17] and Scott, Serfontein, and Seiling[18] have been found in the literature. These both present normal hemoglobin values. However, Taber, Domm, and Davis have reported that estrogens decrease and androgens increase the erythrocyte count in chickens.[19,20]

It would seem that an anemia might be

TABLE 1—Normal Hemoglobin Concentrations in the Blood of Bronze Turkeys

Variety	Age of Birds		Date	Males		Females	
	Weeks	Days		No.	Mean Hb (Gm./100cc.)	No.	Mean Hb (Gm./100cc.)
Standardbred Bronze	24	168	10-13-45	3	11.8	4	11.4*
	26	182	10-27-45	2	13.8	4	13.9*
	27 2/7	191	12-17-45	3	13.7	4	14.6†
	27 4/7	193	1-2-46	4	14.0	6	14.4†
				12	13.3	18	13.7
Crossbred	24	168	10-27-45	1	13.4	3	13.5*
	25	175	11-3-45	1	14.8	3	13.3*
	26	182	11-10-45	1	12.7	3	14.7*
				3	13.6	9	13.8
Broad Breasted Bronze	21	147	11-3-45	7	13.6	6	13.7*
	22	154	11-10-45	4	13.0	2	12.5*
	23	161	11-17-45	4	14.1	2	14.2*
	24	168	11-24-45	4	13.4	2	14.7*
	25	175	12-1-45	4	14.1	2	15.1*
	26	182	12-8-45	4	14.4	2	14.7*
				27	13.7	16	14.0

*Ration—mash plus whole corn; stilbestrol experiments.
†Ration—ground feed only; dimethyl ether experiments.

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expected following estrogen administration to birds. The fact that estrogens induce severe lipemias in birds[21,22] is suggestive in view of the fact that lipemias cause an increased hemolysis of erythrocytes.[23] Further, estrogen-treated chickens are frequently pale in color and have relatively light-colored flesh.[24,25] In turkeys, the normal hemoglobin levels in adult females were found to be only about 75 per cent of the male levels.[17] This type of result has also been reported in adult chickens[26] but not in young chicks.[27] The adult female values in each case are for hens that were either producing or had just finished producing eggs. Since such females have a high level of blood estrogen and blood fat as well as low hemoglobin, some relationship is suggested. Our data bear an obvious relation to the above problems.

MATERIAL AND METHODS

Turkeys of three varieties (Standardbred Bronze, Broad Breast Bronze, and a cross between the two) became available from the college flock at various times in the fall of 1945.

Diethyl Stilbestrol Trials.—Diethyl stilbestrol injections were begun when the birds reached the age of 21 weeks. All three varieties of turkeys were utilized in these experiments, each of which covered a period of about five and a half weeks. The birds had access at all times to the standard turkey mash* employed by the Poultry Husbandry Department and to whole yellow corn.

The diethyl stilbestrol was suspended in soy

*The standard mash had the following composition:

Yellow corn meal	12.0%	Dry milk	10.0%
Ground oats	15.0%	Soybean meal	22.0%
Bran	10.0%	Alfalfa meal	5.0%
Middlings	10.0%	Calcium	1.0%
Meat scrap	14.0%	Salt	1.0%

8 oz. of manganese per ton were added to the above mixture.

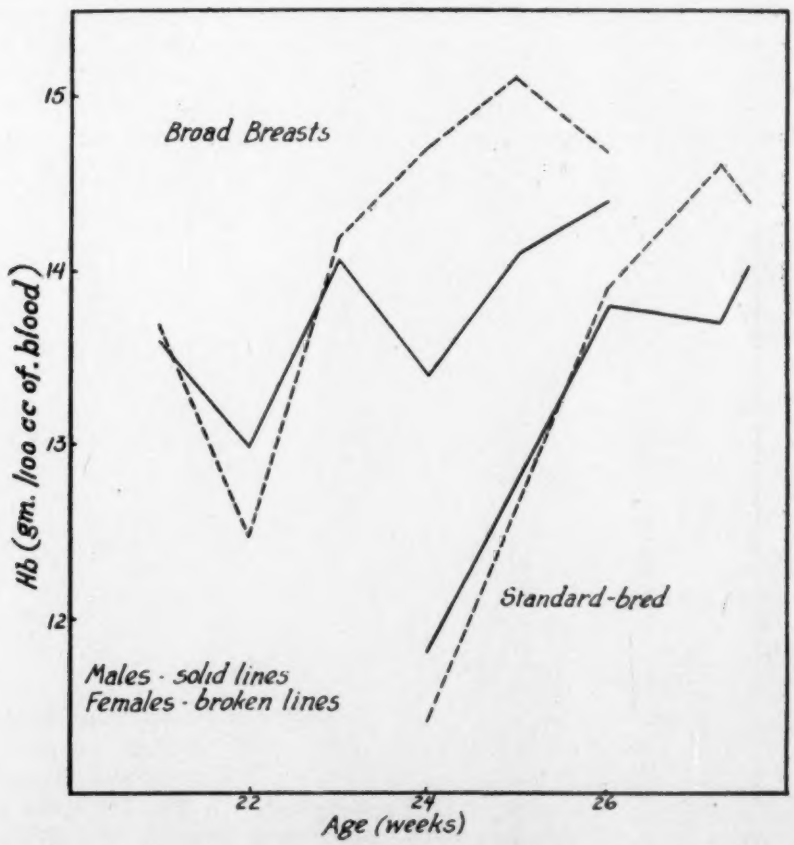

Fig. 1—Weekly variation in hemoglobin concentrations in the blood of normal Standardbred and Broad Breast Bronze turkeys.

oil (250 mg./cc.), emulsified in a mortar and injected into the throat wattle in amounts determined by the body weight of the bird. Since the estrogen was by no means completely dissolved and since the total injection volume was always less than 2 cc., this method may be considered intermediate between pellet implant methods and injection methods. The site of injection precluded early inactivation by the liver. Since the neck wattle is somewhat removed from the general circulation but still definitely vascular, it was hoped that absorption might be relatively slow and prolonged. Since effects of a single injection of 61 mg. in males persisted for at least five weeks (fig. 2), it appears probable that this end was attained. Palpable residues usually remained in this position for more than a week although there was marked variation in this respect. In some cases with a higher dosage, a definite proliferation of connective tissue occurred at the site of injection.

Trials with the Dimethyl Ether of Diethyl Stilbestrol.—The dimethyl ether of diethyl stilbestrol was incorporated in the feed rather than injected. The ration used in the stilbestrol experiments was replaced by an all-mash feed composed of a mixture of ground yellow corn and the regular turkey mash in approximately the ratio consumed by the birds in the stilbestrol experiments. Also, 1 lb. of sardine oil containing the calculated amount of the dimethyl ether replaced 1 lb. of ground corn in each 100 lb. of the final experimental ration. The estrogen comprised 0.01 per cent of the final ration (45.4 mg./lb. of feed). The control ration contained sardine oil without the dimethyl ether. These procedures made it possible to incorporate the estrogen more uniformly in the ration and insured a more uniform intake. The total estrogen intake was then calculated from the feed consumption.

In these trials, the birds were placed on experiment when they reached the age of 23 weeks. Only Standardbred Bronze turkeys were used. The experiments lasted about four and a half weeks.

Hemoglobin Determinations. — Blood samples were drawn from a wing vein and diluted in a 0.1 per cent sodium carbonate solution. Hemoglobin determinations were then run with a Sheard-Sanford photelometer calibrated for mammalian blood. Some difficulties were encountered in the determinations. The diluted blood samples usually contained a small amount of a watery gel. This was removed, either on a small stirring rod or by filtration through glass wool. Blood samples from estrogen-treated birds, however, occasionally showed marked lipemic turbidity. Apparent hemoglobin values from such samples were omitted from our tabulations for obvious reasons. Despite our precautions, it is ap-

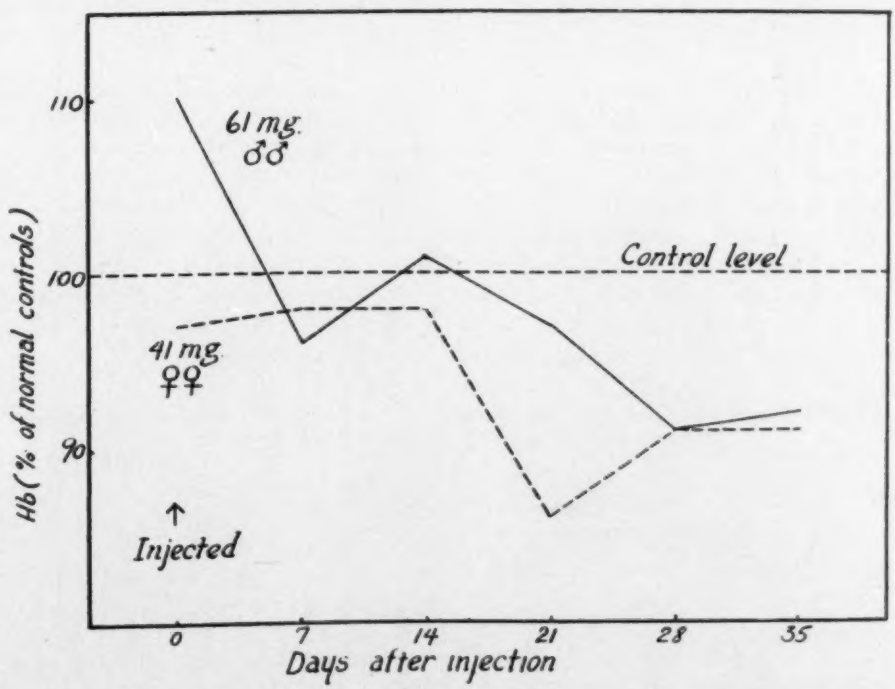

Fig. 2—Hemoglobin concentrations after a single injection of diethyl stilbestrol in Broad Breast Bronze turkeys.

parent that two possible sources of error may have caused our results from these birds to be somewhat too high. First, turbidity due to lipemia may not have been detected. Second, minute clot aggregates may have remained in varying amounts.

Bankowski[16] has criticised the Sheard-Sanford photelometric method for chicken blood because in his hands it gave lower values and was more variable than either the modified Newcomer method or the photelometric acid hematin method. The last criticism may be questioned from his own data. We have not as yet had opportunity to check the validity of his first criticism. Accordingly, these points should be borne in mind in evaluating our results.

RESULTS

Hemoglobin concentrations in the blood of normal controls are shown in table 1. The average hemoglobin level in the blood of normal Bronze turkeys at approximately 26 weeks of age appears to lie between 13 and 15 Gm. of hemoglobin per 100 cc. of blood. Readings from the same bird in successive weeks exhibit considerable fluctuation, however. Thus the single crossbred tom listed in table 1 gave readings of 13.4, 14.8, and 12.7 Gm. of hemoglobin per 100 cc. of blood in successive weeks. Much of this variation is undoubtedly due to inadequacies in the Sheard-Sanford method for estimating hemoglobin. How much is of true biological origin we are unprepared to state at this time. Nonetheless, our values appear to be more uniform than those reported by Rhian, Wilson, and Moxon.[17] During our trials, the hemoglobin level in normal control birds gradually rose. This is plotted in figure 1.

The dimethyl ether of diethyl stilbestrol (dianisyl hexene) has recently been shown to possess an oral potency (measured by oviduct enlargement) two to three times that of diethyl stilbestrol in poults up to 2 weeks of age.[28] At the time of our study, this was unreported. Accordingly, we used the same estrogen level in the feed that has been successful in chickens, namely, 0.01 per cent, a level which proved unnecessarily high. Since poults consume much more feed than chicks, the turkeys to which we fed the dimethyl ether ingested approximately 900 mg. of the estrogen in about thirty days. On autopsy, these birds more frequently showed enlarged and fatty livers than did their controls. Kidney enlargement and degeneration was also noticeable on gross inspection. The hemoglobin level was reduced to about three-fourths of the control values (table 2).

TABLE 2—The Effect of Feeding the Dimethyl Ether of Diethyl Stilbestrol on the Hemoglobin Concentrations in the Blood of Standardbred Bronze Turkeys

Sex	Dimethyl Ether of D.S.		Date	Control Hb (Gm./100cc.)	Experimental		
	% in Feed	Total Dose			No. Birds	Hemoglobin (Gm./100cc.)	(%)
Male	0.01	822 mg.	12-17-45	13.7	2	10.5	76
	0.01	947 mg.	1-2-46	14.0	5	10.7	77
Female	0.01	822 mg.	12-17-45	14.6	2	8.3	57
	0.01	947 mg.	1-2-46	14.4	7	11.8	82

Duration of feeding period—12-17-45 birds, 29 days.; 1-2-46 birds, 31 days.
Age of birds at end of period 12-17-45 birds, 191 days; 1-2-46 birds, 193 days.

TABLE 3—The Effect of Injection with a High Level of Diethyl Stilbestrol on the Hemoglobin Concentrations in the Blood of Standardbred Bronze Turkeys

Sex	Diethyl Stilbestrol		Date	Age Days	Control Hb (Gm./100cc.)	Experimental		
	Mg./Kg.	Total Dose				No. Birds	Hemoglobin (Gm./100cc.)	(%)
Male	56		9-22-45	147				
	56		9-29-45	154				
		825						
			10-13-45	168	11.8	2	7.2	61
			10-27-45	182	13.8	2	9.6	69
Female	56		9-22-45	147				
	56		9-29-45	154				
		569						
			10-13-45	168	11.4	4	8.0	70
			10-27-45	182	13.9	4	10.7	77

Slightly smaller amounts of diethyl stilbestrol injected into the neck wattle of the same variety of turkey produced an anemia, if anything, more severe than that produced by feeding its dimethyl ether (table 3). When the injection was reduced to 61 mg. of diethyl stilbestrol in male Broad Breasted Bronze turkeys of the same age, hemoglobin values fell only about 10 per cent below the control level (figure 2).

DISCUSSION

Our data demonstrate that in turkeys a rather marked anemia may result from large dosages of estrogens, both with daily oral administration continued for a period of four weeks (table 2) and with subcutaneous injection of two massive doses a week apart (table 3). Whereas the hemoglobin values in the feeding experiment were determined at the end of the feeding period, the low values in the massive injection experiment persisted for at least four weeks after the second injection. Also, in the last experiment, where the effects of a single, much smaller dose were followed, nearly four weeks elapsed before maximal depression of the hemoglobin level was observed (fig. 2). It is apparent, however, that the turkey is relatively resistant to an estrogen-induced anemia since the effect of single injections, containing as much as 41 mg. (females) and 61 mg. (males) of diethyl stilbestrol, was not great (fig. 2).

There was some evidence in our data from the stilbestrol-treated birds to suggest that estrogen administration alters their nutritional status. When normal young turkeys are allowed free choice of both whole corn and mash, as the birds grow older, they consume a higher proportion of corn and a lower proportion of mash. Thus, the ratio of corn to mash consumed increases as the birds reach maturity.[29] Following the injection of large amounts of diethyl stilbestrol, however, the ratio for the entire experimental period was 0.61 lb. of corn per pound of mash as compared with a ratio of 1.28 for the control group. Whether this change in feeding habits of the birds is related most directly to their shifted hemoglobin balance or to other metabolic changes induced by the estrogen is unknown. The addition of brewer's yeast to the ration of birds fed the dimethyl ether failed to improve the hemoglobin level, however.

CONCLUSIONS

1) The normal hemoglobin concentration (determined by the Sheard-Sanford photelometric method) in three varieties of Bronze turkeys between the ages of 21 and 28 weeks lies between 13 and 15 Gm. per 100 cc.

2) The dimethyl ether of diethyl stilbestrol administered orally in large amounts reduces the hemoglobin concentration in the blood of turkeys.

3) Diethyl stilbestrol suspensions injected into the neck wattle produce a marked anemia with large dosages and a mild anemia with smaller dosages.

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